Ammonia generation in the gut and the influence of lactulose and neomycin: review of the literature and experimental studies in the rat

AMMONIA GENERATION IN THE GUT AND THE INFLUENCE OF LACTULOSE AND NEOMYCIN

Review of the literature
and experimental studies in the rat

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To Mieke
To my parents

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Chapter I

INTRODUCTION

I.1. Outline of the thesis

These studies and the review of the literature in this thesis were undertaken because no clarity exists about the site of ammonia generation in the gut and how lactulose and neomycin, both generally accepted in the treatment of hepatic coma, decrease ammonia generation. Most if not all clinical literature still claims that ammonia generation primarily takes place in the colon where the major part of the bacterial flora is situated.

In the colon bacterial degradation of urea and of luminal contents derived from dietary residue and epithelial exfoliation, results in the production of ammonia and CO_2. The ammonia diffuses by the mechanism of non-ionic diffusion through the colon and reaches the portal vein whereafter it is finally metabolized to urea in the liver. According to the literature degradation of urea is the main producer of ammonia (3), although it is not clear how urea reaches the colon via the ileum. Moreover according to a recent study of Wrong (9), urea is not an important source for faecal ammonia. In a recent study of Vince (5) who investigated the substrate for ammonia production in several strains of bacteria it was concluded that ammonia generation "in situ" is not derived exclusively from urea, but also from bacterial deamination of amino acids, peptides and proteins. The clinical literature still points to bacteria as main producers of ammonia, although several lines of evidence from the biochemical literature indicate that ammonia may be derived from intermediary metabolism in the gut (13,14,15). Both the small and large bowel are capable of generating ammonia in the gut wall. The chief precursor for this ammonia generation is glutamine, which may be derived from the blood

and from the gut lumen.

Efforts to explain the effects of neomycin and lactulose by their action on bacteria are far from convincing. Neomycin is known to exert its action mainly on aerobic bacteria, especially the enterobacteriaceae. They constitute only one percent to one promille of the total gut flora. It is difficult to conceive that manipulation of this small part can result in a significant decrease of ammonia production.

Several potential effects of lactulose have been described to explain its action, all of which deal with interference of bacterial action. These explanations do not take into account the suggestions from the literature reviewed, that a large part, possibly 75%, of the ammonia production is not bacterially mediated.

Studies were initiated in this direction trying to answer the following questions:

1. How much ammonia is produced in the small and large bowel and is this ammonia production bacterially mediated or from mucosal origin?
2. How is ammonia generation in the gut influenced by neomycin and lactulose?

In chapter 1 therefore the literature will be reviewed concerning the mechanism and sites involved in the generation of ammonia in the gut. This is followed by a review of the proposed action of lactulose and neomycin. At the same time proof will be sought for in the literature for beneficial effects of lactulose and neomycin on hepatic encephalopathy. Subsequently it will be shown that research in the field of ammonia metabolism is still relevant because ammonia still plays a role in most theories concerning the genesis of hepatic encephalopathy.

In the second chapter the in vitro ammonia production of pieces of small and large intestine and the influence of lactulose and neomycin on this production is described.

In the third chapter in vitro incubations carried out with mucosal cells are described. Mucosa cells of conventional and germ-free rats were used in order

to separate the effect of muscle upon ammonia production from mucosal action and in order to distinguish bacterial and mucosal action.

In the fourth chapter the morphological effects of neomycin on mucosa of the small and large intestine are described.

In chapter five a study is described concerning the influence of intestinal flora modulation in relation to ammonia production in the gut of the rat. Rats were first selectively decontaminated in order to remove the aerobic or anaerobic flora and in addition germ-free rats were colonized with a defined aerobic, anaerobic or aerobic/anaerobic flora. Ammonia concentrations in the portal vein were related to this gut flora modulation. The effect of neomycin and lactulose on the luminal and gutwall associated flora related to ammonia concentrations in the portal vein of small and large intestine are described in chapter VI. Portal arterial differences (P-A) for amino acids and ammonia across the intestine in conventional and germ-free rats are described in chapter VII together with the influence of neomycin and lactulose on the P.A. differences.

In the last chapter a comprehensive review of the experimental data is given and put into perspective against data from the literature.

I.2. The origin of ammonia in the gut

The word ammonia is derived from the Greek " Αμμων " used in Libya as an epitheton for Zeus near whose temple the ammonium salt is said to have been prepared from Camels dung.

Ammonia exists in two forms, in solution as gaseous ammonia and as ammonium ion as shown in the following formula:

$$NH_3 + H^+ \underset{\text{<----}}{\text{---->}} NH_4^+$$

Gaseous ammonia is capable of diffusing freely through cell membranes, but the polarized ammonium radical does so only with difficulty and at the expense of energy. In this chapter ammonium refers to NH_4^+ and ammonia gas to NH_3.

The equilibrium constant for ammonia is 9.5. At the pH (fig. 1) of extracellular fluid and arterial blood (7,4) 1-3% of ammonia is in the non-ionized form and 97-99% is in the ionized form.

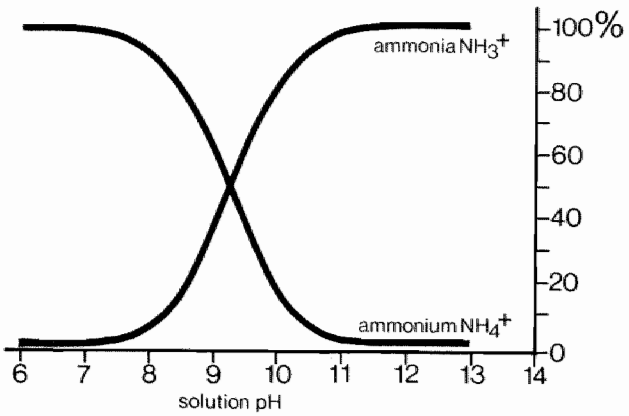

Figure 1: Fraction of ammonia and ammonium ion as a function of pH.

The partial pressure of ammonia in the intracellular and extracellular fluids, which is largely a function of pH, determines the movement of ammonia between these compartments. Diffusion occurs from areas of greater pressure to those of lesser pressure. Every factor that increases the pH gradient between the extracellular and intracellular fluid favours the passage of ammonia into the cells. In the intestine transfer of ammonia from the mucosal to the serosal surface against a concentration gradient is observed in the ileum but not in jejunum (1). It is suggested that ammonia is absorbed in the ileum by active ionic transport. In the human colon however studies clearly indicate that non-ionic diffusion is operative in ammonia absorption (2).

It is generally accepted that intestinal ammonia is produced by enzymatic breakdown of protein metabolites and urea. As a result of the finding that about 13 mM (7.8 g) of urea is daily broken down to ammonia in the intestine of healthy human volunteers (3), it has been suggested that urea is the major substrate for intestinal ammonia production. Apart from small quantities of mucosal urease in the stomach and small bowel, intestinal urease is of bacterial origin and is located in the colon (4). Many viable intestinal organisms like Bacteroides spp, bifidobacteria, Clostridia spp, Proteus spp and Klebsiella spp possess urease activity. Others, notably Escherichia Coli (the predominant Gram-negative aerobic bacteria in the intestine of most species), do not, so that ammonia released by these organisms arises from deamination or deamidation of substances other than urea.

Some organisms produce ammonia by both mechanisms. Vince indicated in her study with a faecal incubation system that the source for bacteria is the aminonitrogen of faecal protein, polypeptides and amino acids (5). She casts doubt on urea as the intermediate source of faecal ammonia. Two separate studies in man, using intestinal perfusion have shown that the large intestinal mucosa is virtually impermeable to urea (6,7) and the amount of urea entering from the ileum is known to be insufficient to account for more than 10% of the rate of urea destruction in man (8). The role of urea as a pre-

cursor of faecal ammonia is therefore in considerable doubt. In a recent study of Wrong (9) it was demonstrated that endogenous urea is not, as has generally been assumed, the major source of faecal ammonia. The endogenous urea pool was labeled in two human subjects with ^{15}N-urea given as a constant intravenous infusion and constant production of endogenous (^{14}N urea) was ensured by identical hourly meals throughout the study. After the first intestinal transit several samples of stool were obtained from both subjects and enrichment of ammonia nitrogen was measured in samples of faecal dialysate, ultracentrifugate, and 48-hours faecal incubations. Expressed as a percentage of plasma urea (^{15}N) labeling, the labeling of stool was very similar in the two studies averaging 8.54 $\pm$ 1.33 % of the various samples of faecal ammonia and significantly less ($p < 0.05$) at 6.88 $\pm$ 0.68 % for faecal total nitrogen.

The labeling of oral salivary protein and plasma albumin was much less showing that faecal ammonia labeling was not derived from alimentary secretions or plasma proteins. By exclusion, faecal ammonia must be mainly derived from other nitrogen sources in the large intestine which are chiefly the residues of dietary protein, intestinal secretions and shed epithelial cells.

Protein ingestion can also increase blood ammonia. A number of investigators has shown that the administration of protein is followed by an increase in blood ammonia levels in cirrhotic patients. Bessman and Clarke e.a. (10,11) showed that blood ammonia levels increase after various types of dietary protein or blood. Clarke found that a typically diphasic increase in venous ammonia levels occurs following a high protein meal. There appears to be a hierarchy in the degree of "toxicity" of different types of protein. Blood appears to be more toxic than meat which in turn appears to be more toxic than vegetable protein. Various explanations have been offered for the varying toxicity of various proteins. It has been suggested that different proteins may be digested at different rates in the digestive tract and that the products may be absorbed differently.

As explained, ammonia generation occurs in several ways, although the literature still indicates that metabolism of bacteria is the most important (12). Nevertheless recent literature reveals that germ-free animals can also develop hyperammonemia, which may be derived from intermediary metabolism in the gut. Nance and his associates reported that germ-free dogs with Eck-fistulae developed porta-systemic encephalopathy (PSE) with hyperammonemia (13). Furthermore they showed that a blood meal induced the same increment in ammonia concentration. Several other lines also indicate that ammonia may be derived from intermediary metabolism in the gut. In 1973 Matsutaka (14) and co-authors demonstrated that small and large bowel of rats can take up glutamine in vitro and at the same time produce ammonia and glutamic acid. Schalm and Van der Mey found in 1979 that hyperammonemia occurs in liverless germ-free rats (15). Warren and Newton (16) have found increased portal vein blood ammonia levels in germ-free rats. In 1978 Weber (17) found that in dogs the large quantity of ammonia released by the small intestine into the portal blood is equal to that released by the uncleansed colon. In the small bowel, it was apparent that the amount of ammonia released by the small intestine was stoichiometrically equivalent to the uptake of glutamine amide nitrogen from arterial blood. Here glutamine was the most likely source of the ammonia released. Felig e.a. demonstrated that glutamine and glutamic acid in the splanchnic bed are largely taken up by the gut and not by the liver (18).

Metabolism of glutamine by intestinal mucosa.

As indicated in the previous pages, the intestine seems to play a significant role in processing the waste nitrogen from other organs. Oxidation of the glutamine carbon provides an important source of energy for the epithelial cells of the intestinal mucosa where glutaminase is largely located (19). Neptune's work in 1965 (20) showing that glutamine was oxidized to CO_2 by incubated ileal tissue from several animal species, is the first reported study indicating that glutamine may be a preferred substrate for the intestine. More direct clues emerged from arterio-venous different measurements

across the non-hepatic splanchnic organs of dogs (21,22), sheeps (23), rats (14) and men (18). These studies revealed an uptake of circulating glutamine by the combined organs drained by the portal vein. Arterio-venous differences for glutamine in vivo across the tissues drained by the superior mesenteric vein were found to be even larger than those measured across all portal-drained organs, localizing much of the uptake in the small intestine (24). The localization was done by Windmueller with an isolated vascularly perfusate preparation of rat intestine. With L(U-^{14}C) glutamine in the perfusate and from analysis of labeled tissue metabolites he concluded that the largest part of glutamine uptake and metabolism occurred in the small intestinal mucosacells. Malcolm Watford (25) harvested columnar absorptive cells (villous cells) in order to study the metabolism of the chicken enterocyte with special reference to the role of glutamine as a precursor of citrulline. He concluded that among added substrates, glucose, glutamine and glutamate are the preferred fuels for respiration in the chicken. Other studies have confirmed that isolated mucosal cells contain a group of enzymes that can account for the metabolic products of glutamine (26). In order to determine quantitatively the metabolic fate of luminal as well as arterial glutamine Windmueller used autoperfused intestinal segments. He concluded that intestinal segments of rats (non-fasted state) utilize arterial glutamine and glucose at about twice the rates, expressed per tissue weight, observed in fasted animals (27). The utilization rate was not increased when the lumen contained amino acids which are transported by carrier-mediated transport. Therefore, the utilization rate of arterial glutamine is concentration dependent, approximately equal to the rate for glucose, and is not increased during carrier mediated transport of substrates which are not metabolized and reduced by fasting.

In the same preparation he measured the radio-active products of L-(U^{14}C) glutamine. The carbon was partly incorporated into tissue acid soluble material (14%) and the rest reappeared with little delay in intestinal venous blood in CO_2 (57%), citrulline (6%), proline (5%), ornithine (2%), alanine

(3%) and organic acids (18%) predominantly citric acid and lactic acid. The product distribution of the arterial glutamine carbon was similar in conventional or germ-free rats, precluding the intestinal microflora as a source for any of the metabolic products. The rate limiting step for the glutamine catabolism is apparently its deamidation and not the metabolism of the resulting glutamate since none of the L-($U^{14}C$) glutamate administered luminally escaped metabolism during its transfer across the intestine. The ^{14}C-labeled products of glutamate catabolism were the same as those arising from glutamine (28). The results of Windmueller also indicate that glutamine was metabolized identically whether it entered the mucosal cells across the brush border from the lumen or across the basolateral membrane from the arterial blood, suggesting that a single mucosal pool exists. During digestion two sources of glutamine "compete" for the same enzymes. The presence of glutamine or glutamate in the lumen reduced the rate of glutamine utilization from the blood. However since most of the luminal glutamine is also metabolized, the total rate of glutamine metabolized was increased by 70% when both sources of glutamine were available (28). Therefore he concluded that the utilization rate of arterial glutamine was not reduced by fasting, which was in contrast to the study of Hanson (29), who found a higher glutamine utilization in rats deprived of food.

Windmueller also studied the metabolic fate of the glutamine nitrogen. Nitrogen taken up by jejunum as glutamine could be accounted for by the net release of ammonia, ornithine, alanine, proline, glutamate and citrulline. About 75% of the glutamine amide nitrogen, released by a phosphate dependent glutaminase, could be accounted for as ammonia and the remainder as one of the two nitrogen atoms of citrulline. The glutamine amino group could be accounted for by the remaining nitrogen of citrulline and the other amino acids. Windmueller (26) proposed pathways of glutamine metabolism in rat intestinal mucosa. The first step of catabolism is catalyzed by a phosphate dependent glutaminase. Mucosal scrapings indicate that rat small intestine contains sufficient protein glutaminase to account for the rates of glutamine

breakdown observed. Although other glutamine degrading enzymes have been found the concentrations of these enzymes are too low and insufficient to account for the breakdown of glutamine (26). Distribution of glutaminase was found in the small intestine of all animals he surveyed (rat, dog, cat, hamster, mouse, monkey, rabbit, guinea pig and chicken). Glutaminase distribution is consistent with its proposed role. High activity of this enzyme was found in duodenal, jejunal and ileal mucosa, and relatively low glutaminase activity was found in mucosa from rat stomach, coecum and colon (19). Consistent with this, rat stomach utilizes relatively little vascular glutamine (30). The phosphate dependent enzyme is located in the mitochondria and can be activated by phosphate (19). Activity of intestinal glutaminase in rats was not increased by feeding large amounts of glutamine, nor was it decreased by fasting (19).

In the proposed pathway of Windmueller (fig. 2) other enzymes than glutaminase are needed to support this. The remaining ammonium, which is not released into the portal vein, is converted by carbamoyl-phosphatase. Rat intestine contains N-acetyl glutamate synthase and the co-factor needed for this enzyme is N-acetyl glutamate (31). The glutamate produced in reaction I (fig. 2), is reduced to glutamic acid-γ-semialdehyde which can be further reduced to proline or transaminated to ornithine.

Glutamic acid-γ-semialdehyde is converted to pyrroline-5 carboxylate. The enzyme that converts glutamate to pyrroline-5 carboxylate has been characterized in the mitrochondria from rat intestinal mucosa (32,33). Ornithine carbamoyl transferase catalyzes the conversion of ornithine and carbamoyl-phosphate to citrulline. Citrulline is then released into the portal vein. The intestine appears to be the rare mammalian tissue that can effect a net synthesis of ornithine and protein from glutamate (34,35). If glutamate is not utilized to form proline or to form ornithine via glutamic-acid-semialdehyde, it can be transaminated with pyruvate to alanine.

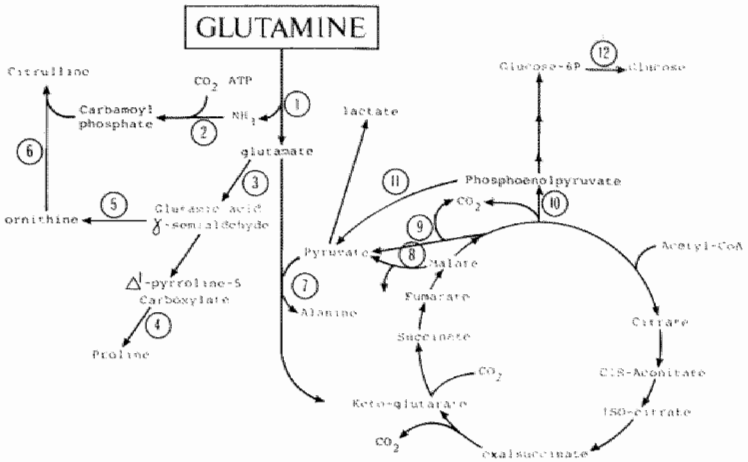

Figure 2: Pathways suggested for glutamine metabolism by rat intestinal mucosa (Windmueller, 26).

The importance of glutamine as a respiratory fuel.

Windmueller indicated the importance of glutamine as a respiratory fuel for the intestinal cells in several studies with rats (24,34,37). In tracer studies he discovered that more than half of the CO_2 produced by intestine of fasted rats was derived from the metabolism of circulating ketone bodies and 36% was derived from glutamine. Although glucose and glutamine were utilized in approximately equal amounts, most of the glucose carbon appeared in alanine and lactate and only 11% in CO_2. Likewise, when in this experiment animals were fed with a diet containing 50% of glucose and 15% corn starch, the contribution remained small.

A specific role for glutamine in the physiology of the animal.

There is little evidence that, apart from its role as a respiratory fuel, glutamine serves a more specific function in the physiology of the intestine. Glutamine has been reported to stimulate the transport of glucose, water and sodium ions by intestine incubated in vitro. Whether this is a specific

function of glutamine is not known but it could be related to its energy producing capacity. As a primary site for metabolizing plasma glutamine, the intestine plays an important role in processing the waste nitrogen produced by other tissues. All of the nitrogenous end products, as revealed in fig. 2 of intestinal glutamine metabolism can serve ultimately as urea precursors. The nitrogen flux along this pathway is large and Windmueller (26) estimates that 30% of the nitrogen utilized for hepatic ureagenesis in non-fed rats is derived from the nitrogenous products of intestinal breakdown. The carbon atoms of alanine, proline and lactate are available for gluconeogenesis in the liver. Citrulline serves as a precursor for arginine in the brain and in the kidneys, which are an important endogenous arginine source for protein synthesis in muscle.

Although it was previously believed that the liver also releases citrulline, recent studies indicate that this is not true (36). Windmueller showed that the kidneys take up circulating citrulline at about the same rate as its release by intestine. No additional significant sites for citrulline release or uptake were revealed by arterio-venous measurements across other major organs (37).

Although the clinical literature still indicates the bacterial flora in the colon as the main producer of ammonia, a review of the literature, especially the biochemical literature does stress that metabolic ammonia generation in the gut is important.

I.3. Lactulose: the mode of action and efficacy

Lactulose is a synthetic water-soluble disaccharide consisting of galactose and fructose in combination as 1,4-β-galactosido-fructose.
The molecular weight of lactulose is 342.3 and its melting point is situated between 169.5-170.4°C.

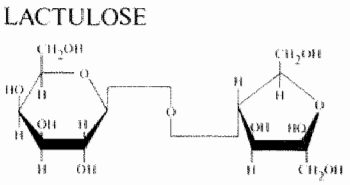

Figure 3: Lactulose

Although lactulose is a disaccharide and is chemically similar to other disaccharides such as lactose, lactulose does not occur naturally and there are no enzymes in man or animal capable of hydrolyzing lactulose (38). Only bacteria contain enzymes that are capable of hydrolyzing lactulose (39). Like other intact disaccharides, unhydrolyzed lactulose is virtually non-absorbable in the small intestine. Experiments in baboons and rats have shown that after ingestion of enormous amounts of lactulose (15-20 g per kg) only minute quantities (10-100 µg per ml) could be detected in the serum (40). The main site of degradation of lactulose must be the colon since relatively few bacteria are found in the small intestine (41). Thus lactulose reaches the colon almost intact and there bacterial degradation begins. Hoffmann e.a. (38) identified lactic acid as the principal metabolite of the bacterial breakdown of lactulose. In addition small amounts of volatile fatty acids and ethanol are formed which acidify the colonic content so that an impressive

decrease in stool pH is achieved.

Potential mode of action.

1. The original hypothesis of Bircher and his associates (42) that lactulose exerted its beneficial effect by changing the bacterial flora of the intestine, has not been supported by subsequent studies. Bircher hypothesized that lactulose enhanced the growth of no urease containing bacteria such as Lactobacilli. In normal subjects given lactulose Flock e.a. (43) were unable to detect significant changes in the pattern of faecal flora during administration of lactulose, nor were any significant changes in the flora pattern observed during treatment of cirrhotic patients. Hoffmann e.a. (39) found significant decreases in total bacterial counts of Escherichia coli and Bacteroides spp with no more than a proportional increase in Lactobacilli and no alteration in faecal ammonia values. In cirrhotic patients lactulose produced clinical improvement one to three weeks before the development of consistent changes in faecal bacteriology and benefit was obtained even in patients whose stools contained no Lactobacilli. Zeeger e.a. (44) observed a significant increase in faecal Lactobacilli during lactulose administration, but found no consistent changes in coliforms or in Bacteroides spp. Conn e.a. (45) found no significant change in the number of Lactobacilli in normal subjects or in cirrhotic patients being treated with lactulose although lactulose administration was associated with a decrease in the total number of aerobic bacteria and in the number of coliform bacteria. Vince e.a. (46) however in a recent report showed a considerable increase in both aerobic and anaerobic species of Lactobacilli. Results however were not consistent in all patients. Counts of E. Coli decreased during lactulose therapy in one patient who responded clinically and in one who did not. Counts increased dramatically in another who responded clinically. Counts of Bacteroides spp increased during lactulose therapy in one and decreased in the other both of whom responded well clinically due to lactulose

treatment. Therefore results were difficult to relate to the change in flora. The contradictory results in these studies tell us very little about the role of intestinal organisms in the induction or recovery of P.S.E. Also none of these investigators studied the effect of lactulose on the gut-wall associated flora in several locations of the intestine. It still leaves us with the question: are Lactobacilli or E. coli of crucial importance or the total number of aerobic and/or anaerobic bacteria?

2. A second mechanism by which lactulose may act is through trapping ammonia in the acidified content of the bowel. In the lumen of the gastro-intestinal tract acidification increases the pH gradient between gut content and mucosa and reduces ammonia absorption from the bowel. Summerskill e.a. (47) published a report on quantification of ammonia concentration and output in the upper gastro-intestinal tract in health and in patients with diseases of the liver. High concentration gradients between the gastro-intestinal tract and the blood were found under basal conditions. Most of the findings in this respect were consistent with non-ionic diffusion of ammonia due to the relative pH difference between opposite sides of the gut membrane. Castel e.a. (48) demonstrated that absorption of ammonia from the isolated colon varies directly with the pH of the perfusate. Bircher e.a. (49) showed in dogs that reduction in pH of the lumen resulted in reduction of the ammonia concentration of venous blood leaving the colon and found that pH levels of less than 6.2 reversed the absorption of ammonia. Several studies have shown a fall in stool pH (39,50) due to lactulose treatment, although only one study has been carried out in humans in vivo. This study was carried out by Bown (51) who used a radiotelemetry capsule and found a fall in the pH of the colonic contents in humans. The lowest level was observed in the right hemicolon. When the low pH enhances the excretion of ammonia in the colon, loss of ammonia should be detected in the faeces. Studies on

ammonia loss however did not show the expected increase (44,52). Agostini e.a. (52) used other cathartics besides lactulose (sodium sulphate, mannitol and sorbitol) but he did not observe an increase in the concentration of faecal ammonia either. He claimed that despite the increase in the quantity of faeces no increase in the absolute amount of ammonia was observed in the faeces due to lactulose treatment, although the other purgatives did show a modest rise. Vince e.a. (53) claimed that a decrease of the pH reduces ammonia both by lowering production and absorption. In her study on ammonia production by intestinal bacteria a significant decrease in count of the enterobacteriaceae at low pH was noted. Ammonia production by Escherichia coli decreased significantly when the pH was lowered from pH 7.2 to pH 4.6. Considerable variation was noted in the ability of different species to produce ammonia. Experiments with cultures containing both Escherichia coli and Proteus Mirabilis showed that more ammonia was produced at low pH, than was produced by cultures of single species.

Although less ammonia was produced at low pH, a still significant amount of ammonia was generated at pH 5.0. When using a faecal incubation system, Vince noted that acidification to 5.0 or less with hydrochloric acid or a lactic acid mixture, significantly reduced ammonia generation from faecal cultures. Whether this pH lowering effect to 5.0 or less significantly contributes to a decrease in the production of ammonia in the lactulose treatment is difficult to say because a pH lower than 5.0 after lactulose treatment was only measured in the coecum (51) in the study of Bown, whereas in the rest of the colon the pH amounted to 6.7 and 6.6 respectively.

3. A third mechanism by which lactulose may act is via the catharsis which may develop due to acid products resulting from the breakdown of lactulose. It is known that acid has a cathartic effect on the intestine. Lactic acid in Krebs solution, which reduced the luminal pH to 3.6 caused

enhanced fluid propulsion and increased circular muscle contractions (54). Indeed after lactulose administration these effects, acid stools and increased bowel activity proportional to the amount of carbohydrate ingested, can be the consequence of control substances such as sorbitol and magnesium sulphate. They both produce an acid stool, whereby sorbitol is known to be converted to organic acids by bacteria. However both mannitol and sorbitol do not appear to produce clinical improvement in hepatic encephalopathy (52).

4. A fourth mechanism was proposed by Vince (53). The presence of carbohydrates facilitates the growth of bacteria and the incorporation of nitrogen into bacterial protein. Vince suggested that lactulose actually stimulated incorporation of ammonia into bacterial protein. In her study she tested this phenomenon by incubating lactulose and ammonia with several strains of bacteria. During the final 6 hrs, ammonia disappeared completely from all lactulose-incubation systems. Administration of other substrates like glucose, mannitol and sorbitol also caused a fall in ammonia concentration, similar to that observed with lactulose. Vince has therefore claimed: 1) preferential use of lactulose as a carbon and energy source would exert a sparing effect on the metabolism of the bacteria, resulting in a decrease of the production of ammonia, liberated normally as byproduct during metabolism; 2) a readily available energy source would presumably encourage assimilation of ammonia, an energy-requiring process which may not occur so readily under conditions of carbon limitation.

It is not clear how these four mechanisms integrate. Furthermore none of these mechanisms takes the non-bacterial ammonia production in the gut into consideration (chapter I.2.).

I.4. Lactulose in hepatic encephalopathy

I.4.1. Uncontrolled trials

Most of the studies done in the early days were devised to study whether lactulose had any demonstrable effect. None of these studies attempted to evaluate the response to oral lactulose by introducing a control treatment period. These studies unfortunately contained few patients and even individual cases. However the results do suggest that it was possible to increase protein intake and that lactulose did improve mental state in patients with chronic portal-systemic encephalopathy.

Markhoff (55) reported a case of a 50 year old male suffering from portal-systemic encephalopathy, who became conscious six days after beginning with lactulose therapy. Writing tests reverted to normal and his arterial ammonia fell drastically.

Palma (56) reported a study of 5 patients to whom lactulose was administered immediately postoperatively in patients having undergone a portocaval shunt. Clinical and hematological findings were evaluated in comparison with those observed in another 20 patients having undergone the same surgical operation. Lactulose made rapid bowel movements possible. Ammonia levels 48 hours after the shunt were always lower than mean values in untreated subjects and remained within normal range after resumption of oral diet and the increased protein intake. Palma showed excellent results, although the number of patients was limited.

Lande and Clot (57) reported a study of 12 patients with liver cirrhosis. Ten patients responded well to the therapy. Two did not. They concluded that lactulose is very effective in the therapy of hepatic coma.

Rottiers (58) reported a study in which patients were divided into three groups. Group A consisted of five cirrhotic patients having a portacaval shunt who were hospitalized with the clinical picture of hepatic precoma or coma and received lactulose as only treatment at a dosage of 150 ml/day.

Group B consisted of six cirrhotic patients without surgical portacaval shunt, who were hospitalized with the clinical picture of hepatic coma.
Group C consisted of four cirrhotic patients who did not show symptoms of hepatic coma and in whom the development of the fasting ammonemia following a 3 day course of 150 ml lactulose syrup daily was studied. Moreover in three of them an ammonia tolerance test was carried out before and after the same administration of lactulose syrup daily, also for three days.
In the first group of cirrhotic patients there was an obvious improvement of the clinical condition with lactulose treatment in all five cases. The results were different in the second group of cirrhotic patients. In two cases the results were very good. In one case the result was insignificant. One died, one had heart failure so that the result was difficult to assess. In the last case, there was improvement with a higher dosage. In the third group, the fasting blood ammonia levels dropped significantly after a three day course of lactulose in three out of four cases. As to the three ammonium-chloride tests, the following can be stated. Only one showed a clear difference, both regarding the clinical manifestation, the blood ammonia levels and the EEG after lactulose administration. Rottiers therefore concluded that the best indication for the treatment with lactulose seems to be the chronically recurring encephalopathy, which occurs in relatively well stabilized cirrhotic patients, mainly in those with a surgical portacaval shunt.
Fung and Khoo (59) studied two patients with chronic recurrent hepatic encephalopathy. They were used as their own control, since their hepatic encephalopathy was first treated in the conventional way, using neomycin syrup, protein restriction and colonic washouts and then lactulose therapy alone (30 ml three times a day) without protein restriction in their diet. Lactulose therapy produced marked improvement with disappearance of flapping tremor and constructional apraxia. The patients became fully conscious. This improvement occurred in spite of 70 to 80 gr of protein per day in their diet. There was also a clear improvement in the EEG recorded during lactulose therapy.

Demeulenaere (60) reported in 1969 a similar study. He added however an additional group to his study, in which he gave cirrhotics a lactulose enema. Although he stated that results were correlated with EEG, ammonia level and clinical signs, results demonstrated that these parameters were only determined occasionally. He concluded that prolonged administration of lactulose in lucid and active cirrhotics is a very useful and inexpensive therapeutical modality. Also rectal administration of lactulose improves the mental condition and sometimes spectacularly.

Helms (61) reported a study of 10 patients. Significant improvement was reached in two patients.

Combes (62) evaluated long term protein tolerance in portal systemic encephalopathy and showed an increase in tolerance in patients to whom lactulose was given for long periods.

Gauthier (63) reported in 1970 a study of 37 patients with daily doses of 90 to 150 ml/day. He had succes in 27 cases.

Geffroy (64) reported a study of 20 patients. 18 Patients responded well to therapy with lactulose. Mental state and ammonia levels were used as parameters. Geffroy concluded that it was possible to increase protein intake together with lactulose-therapy. In patients responding favourably to therapy, the pH of the faeces fell to 6.0.

James (65) reported a study of patients in which cerebral blood flow and cerebral metabolism were studied in order to evaluate porto-systemic encephalopathy after a 10 day course of lactulose. As a result of therapy five out of the six patients showed clinical improvement. There was a mean increase in cerebral oxygen utilization but no changes in either mean glucose consumption or in mean cerebral blood flow.

Kardel (66) evaluated lactulose treatment in eleven patients, the majority of whom were treated for four weeks. Only three patients improved clinically on lactulose, all of whom showed an improvement in EEG tracings. The improvement occurred within two to five days and withdrawal of the drug was followed by deterioration. One patient who did not improve clinically, nevertheless had

improved EEG recordings. Blood ammonia was not affected by lactulose in this study. No mention of dietary protein was made and blood ammonia was not measured during the fasting state. The author stated that lactulose seemed to work best in patients with more severe encephalopathy.

Almici (67) reported a study of 19 cases with advanced hepatic cirrhosis with 1st to 3rd stage coma and grade A to B EEG alterations. Clinical, EEG and blood ammonia normalization was noted in 16 cases. In 5 cases it was also possible to carry out diuretic treatment in order to reduce ascites.

Eisenbury reported in 1974 a study of 14 cases in which 10 were treated succesfully with lactulose (68).

Reikowski treated 36 patients with cirrhosis of the liver (69). Virtually all patients responded well to lactulose treatment and ammonia levels fell.

Seidlova reported a study of 14 patients with cirrhosis of the liver (70). The evaluation was carried out according to clinical criteria and EEG examination. It was possible to increase the dietary protein intake and acidification of the faeces occurred regularly. Eleven patients responded well to the therapy.

1.4.2. Controlled trials

In the better trials, every effort was made to adequately control variables which might influence the response or assessment of results.

Ma et al (71) conducted a long term trial of lactulose in ten patients with chronic portal systemic encephalopathy. Improvement was assessed by neurological state and EEG. The patients previously required neomycin and protein restriction. Wherever possible the trial was divided into five phases.

1. Observation in the hospital for one week while the usual regimen of treatment with neomycin and protein restriction was continued.
2. Observation in the hospital for a further three weeks during which lactulose was substituted for neomycin.
3. Observation after discharge from the hospital when treatment with lactu-

lose was continued.

4. Readmission to the hospital and observations of the effects of withdrawal of lactulose.
5. Reintroduction of lactulose when signs of encephalopathy developed.

Six out of 10 patients had a satisfactory response and were maintained on lactulose for up to twelve months. Two patients failed to respond to either lactulose or neomycin and two patients withdrew from treatment due to intolerance to the drug. Four out of six who had improved on the drug were withdrawn from therapy and deteriorated both on EEG and clinical grounds, but subsequently improved when the drug was reintroduced. Ma indicated that the drug is effective in controlling chronic portal systemic encephalopathy and is a useful substitute for neomycin.

Rorsman (72) and co-authors using lactulose in a long-term study could increase the protein intake in three patients. The response was based on clinical and EEG evidence of improvement following controlled exacerbation of encephalopathy. All three patients had previously been treated with protein restriction but only one had received neomycin. In all cases it was possible to start the trial with a period of protein restriction. If the condition returned to normal an exacerbation was induced by increasing the dietary protein. With protein intake on this higher level, lactulose was started, usually in a dose of 20 ml three times daily. Administration of lactulose produced a regression of the clinical symptoms during increased protein intake as well as a return of the mean frequency of the EEG to about the same stage as before the dietary protein was increased. Withdrawal of lactulose was associated with encephalopathy. The optimal effect of lactulose was evident in 10 to 14 days. When the initial trial had been completed the patients were discharged with dietary instructions implying moderate protein restriction and continuation of lactulose. Two patients remained well for more than 2 years, one on a low protein diet, the other on moderate protein restriction. The third died, despite protein restriction and neomycin.

Siebner (73) found that of twelve patients treated with lactulose eight showed a superior effect with lactulose as compared to broad spectrum antibiotics, while three had equivalent effects. One patient derived no benefit from lactulose.

In 1970 Zeegen et al (74) reported the clinical and biochemical changes associated with lactulose therapy in seven patients with chronic portal systemic encephalopathy and compared the results with a magnesium sulphate solution. Those who responded appeared to have a higher degree of encephalopathy and higher initial blood ammonia levels than the 4 non-responders. Results were assessed in a non-blind manner by a range of clinical and biochemical tests. The tests included EEG, measurement of mental state, blood ammonia level, faecal pH, weight and frequency. The 7 patients had undergone shunt operation. They were initially hospitalized for 7-14 days when a protein intake of 60 to 80 g/day was started and maintained. Hereafter alternately lactulose or magnesium sulphate was given and in two patients magnesium sulphate was the initial treatment. Magnesium sulphate was used to produce a similar quantity of stools. During the initial control period there was little change in the degree of encephalopathy. Only 3 of the 7 patients derived obvious clinical benefit when given lactulose and deteriorated when transferred to magnesium sulphate. No patients responded to magnesium sulphate. Two of the 4 patients who did not respond to lactulose showed clinical improvement with neomycin and were discharged with this drug. Changes in the lactulose period were paralleled by improvement of EEG tracings. There was also a significant fall in arterial blood ammonia levels in all 3 who responded, but also in 3 of the 4 non-responders. Faecal pH was not related to clinical response and the most acid diarrhoea was produced in 2 non-responding patients. It was concluded that lactulose was of value in certain cases of chronic encephalopathy, and was a useful alternative to neomycin although not always interchangeable.

In 1971 Fung (75) presented a long term study of 11 cases of hepatic encephalopathy treated with lactulose. There was improvement in 9 patients but this

could be attributed specifically to lactulose alone in only 6 patients. Seven of the 11 patients had evidence of chronic hepatic encephalopathy and were given lactulose on a long-term basis. Five of these 7 cases had prior neomycin therapy, thus enabling comparison of lactulose with neomycin therapy. Sustained long-term improvement was seen in only 5 of the 7 cases. In these 5 cases, lactulose was more effective than neomycin, since it abolished all evidence of hepatic coma. In the face of a normal protein intake one of the 5 succumbed to bleeding. The conclusion was that lactulose is useful and effective in the treatment of both acute and chronic encephalopathy and is more effective than neomycin.

In 1971 Imler (76) reported a study of 18 hospitalized cirrhotics with spontaneously appearing and persistent episodes of portal systemic encephalopathy. Only one patient had a surgical portacaval shunt. All patients were first started on a protein-restricted diet before other treatment was initiated. Lactulose was compared to neomycin, chlortetracyclin (1 g i.v.) or Lactobacillus acidophilus. No patient responded in any way to the Lactobacillus acidophilus treatment. Two patients however showed rapid improvement when neomycin was given. Lactulose treatment was first given to 4 cirrhotics at a dose of 50 ml per day, which gave only a slight improvement in one patient. The best response to lactulose occurred in those given 90 ml daily. When administered to 10 patients, 7 showed marked improvement on the basis of clinical and EEG evidence. When the dose was increased to 135 ml daily to the three patients who did not respond, one showed a slight improvement, but another deteriorated with diarrhoea and hypokaliemia. In seven patients, lactulose treatment was compared with neomycin or chlortetracycline. All patients responded better than with lactulose. The ammonia levels and EEG findings correlated well with clinical improvement. It was not mentioned however whether the treatment was instituted at random and whether the results were evaluated under blind conditions.

Bircher (1966, 1971) (77,78) reported a carefully designed within-patient trial with six patients. The 6 patients included in the analysis of the

results had far advanced cirrhosis. One patient was an alcoholic, but the origin of the cirrhosis in the others was unknown. Two patients had a surgical portacaval shunt. Of the 17 patients started in the trial, only 6 fullfilled the criteria at the end of the study period [(coma grade III to IV scale 0-V) within 3 days after withdrawal of neomycin or lactulose with 40 g protein a day, no change in state of liver disease during the study, absence of azotemia and uncorrected electrolyte imbalance, absence of gastro-intestinal hemorrhage within one month prior to and during the study, diuretic therapy kept constant]. The investigation in each patient lasted 4 to 5 weeks and was divided into 4 treatment periods:

1) Lactulose

2) Neomycin

3) A laxative (sorbitol or magnesium sulphate)

4) Lactulose + higher protein intake.

The treatment period lasted 7 to 10 days except for the laxative period which had to be terminated earlier because all patients developed coma. Lactulose dosage ranged from 90 to 150 ml/day (67 g/100 ml). Neomycin was given and varied from 2-8 g/day. The laxatives were given in doses adjusted to produce the same degree of laxative effect as lactulose. All patients did well on neomycin and protein restriction to 40 g per day. They also did well on lactulose and no patients showed signs of deterioration when protein intake was increased to between 70 and 100 g/day in the second lactulose period. Tolerance to increased protein was not tested when neomycin was used. The six patients continued to take lactulose without dietary protein restriction and symptoms of encephalopathy remained well controlled in all. Two patients discontinued lactulose intake themselves. One of them had to be rehospitalized in coma, but promptly responded to resumed lactulose treatment. No side effects of long term lactulose treatment could be detected. Five patients died after six weeks to ten months. The causes of death were oesophageal variceal bleeding renal failure, cerebral hemorrhage and unexplained sudden death. Consecutive bacteriological studies of the stools during the whole

investigation period were available for two patients. It was concluded that there was no correlation between the clinical effect of lactulose and changes in faecal bacterial composition. Bircher concluded from the effects of lactulose and neomycin, that there was no appreciable difference between the therapeutic effects of neomycin and lactulose. The control "laxative" solution (sorbitol or magnesium sulphate) was without effect.

In 1973 Rodgers (79) reported a long-term study of six patients who were originally entered into the study but only three were followed for a year or longer. He used lactulose (50%) and sorbitol (60%) in order to compare these drugs in the management of hepatic encephalopathy in a double blind study. Patients were instructed to consume a 50 g protein diet. During the first year of the study patients were continued treated with one medication for a period of two months and then switched to the other for a similar length of time. During the second year a controlled period separated treatment periods. EEG, blood ammonia and clinical state were observed. It was concluded that both lactulose and sorbitol were beneficial in the treatment of hepatic coma. Whether lactulose or sorbitol was superior could however not be concluded from this study. In contrast to many investigators, Rodgers observed a reduction in stool pH after sorbitol treatment. It was also difficult to relate clinical state with EEG and blood ammonia levels.

In 1981 Orlandi (80) reported a randomized study in order to compare the course of hepatic encephalopathy in patients treated with neomycin plus magnesium sulphate or with lactulose. One hundred seventy three patients were selected with a diagnosis of cirrhosis. Patients received orally neomycin tablets of 1 g four times a day and purgation with 30-60 g of magnesium sulphate orally was achieved in patients with grade I hepatic encephalopathy. Two grams of neomycin four times a day and magnesium sulphate (30-60 g) were given in grade 2 or 3 of hepatic encephalopathy. Ten to 35 ml of a 50% lactulose syrup were given three times per day orally. The aim of the therapy was to induce two bowel movements a day. Mean values of duration of the treatment with neomycin and lactulose were respectively 17.3 and 18.6 days in

grade I and 13.3 and 16.2 days in grades II and III hepatic encephalopathy. Grade I was defined when three or more of the following signs were present:

1. slow or slurred speech,
2. disturbances of memory,
3. abnormalities of behavior and/or mood,
4. abnormalities of gait,
5. asterixis,
6. writing difficulty,
7. abnormal performance of serial subtractions,
8. difficulties in drawing a five pointed star,
9. disturbances in Reitan trailmaking test A en B (81),
10. EEG changes.

Grade II includes major changes of mental state, somnolence and loss of discrimination. Grade III is coma, with inability to test mental state. Improvement of the therapy was transition from grade 1 to grade 0 or from grade 2 or 3 to grade 1 or 0. The outcome was defined as unsatisfactory when transition to a less severe grade did not occur within 14 days. At the time of randomization 114 of the patients exhibited grade I encephalopathy. Fifty two were in grade II and 7 subjects were in grade III. Eighty two received neomycin, 91 lactulose. Forty nine patients with grade I hepatic encephalopathy were allocated to the neomycin group and 65 to the lactulose group. The respective values were 33 and 26 for patients with grade II or III hepatic encephalopathy. Transitions from grade II and III to grade I or 0 showed a difference in favour of neomycin. However the study demonstrated a similar course of the symptoms when patients with grade I hepatic encephalopathy were treated with neomycin sulphate or with lactulose. Improvement in EEG pattern was observed in most patients. Also plasma ammonia decreased in the neomycin and lactulose group. Stool pH decreased only in the lactulose group. The drug of choice in long-term treatment of hepatic encephalopathy should be lactulose because of the benefit risk ratio of neomycin which has many side effects. Although the study was large and excellent in set up, no double blind design

was used, this however is very difficult to achieve because of the specific effects of the drugs under investigation.

1.4.3. Controlled double blind trials

Simmons (1970) (82).

Twenty six patients with hepatic encephalopathy were selected for the study. All had a history of excessive alcohol consumption and in 16 alcoholic liver disease was confirmed histologically. After being accepted into the study, patients were randomly assigned to one of two groups according to the next available code number. The nature of the code was not known to the investigators. One group received lactulose, the control group received a substance similar in flavour and appearance but containing glucose. The duration of the treatment was 10 days. All patients initially received a 40 g protein diet but after 5 days dietary protein was increased to 60 g in those patients who were free of encephalopathy. Five patients (3 in the lactulose group and 2 in the glucose group) were eliminated from the study, mainly because of complications of their hepatic disease, unrelated to encephalopathy. On the basis of improvement in encephalopathy the lactulose group showed a superior response to the glucose group: a result which was statistically significant. EEG's were not performed. Fasting venous blood ammonia levels did not change significantly. After 10 days however, the patients receiving lactulose had a mean ammonia level which was significantly lower than patients receiving glucose. This double blind study strongly supports the value of lactulose in the treatment of hepatic encephalopathy.

Brown (1970, 1971) (83,84) reported a long-term double-blind within-patient trial in 20 patients. They were initially hospitalized for a week on a diet containing 20 g protein per day which was gradually increased until neurological signs of encephalopathy were evident. 9 of the 20 patients were then admitted to a double-blind trial to receive randomly either lactulose or sorbitol for varying periods on an outpatient basis. Of the other 11 pati-

ents, 3 were uncooperative in taking lactulose and were eliminated from the study. Two had only been under treatment for a few months. Three were maintained on a protein restricted diet only and 3 died early, 1 from lymphoma and 2 from acute alcoholic hepatitis. Of the nine patients one did well on sorbitol, the other did well on lactulose only and was discharged on a 100 g protein diet. The other 7 patients were given alternating periods of lactulose or sorbitol syrup. Five patients responded well to both drugs. Blood ammonia levels were not strikingly reduced and did not always correlate with improvement. The EEG changes, although not impressive, returned to normal. Both lactulose and sorbitol produced a laxative effect which was associated with a fall in faecal pH. Brown concluded that lactulose was effective in outpatient management of hepatic encephalopathy. Sorbitol given as control, often produced a response but was judged much less effective than lactulose. It is a pity that dietary protein was not kept constant in this study.

Germain (1973) (85) performed a double blind study in 18 patients with chronic PSE after portocaval anastomosis in which he compared lactulose and sucrose. Nine of these 18 were randomly selected to have each form of therapy in a simple comparison design. The patients were evaluated before and after 15 days of therapy with 60 ml of lactulose or sucrose daily. Patients in both groups had mild PSE, and only a few patients showed elevations of venous ammonia concentration. EEG's showed mild to moderate changes. The investigators showed that clinical manifestations and blood ammonia were not significantly different in the patients receiving lactulose and in those receiving placebo. However, lactulose had a significant effect on psychometric test abnormalities and on EEG disorders. Although there was a reasonably good correlation between the derangement of mental state and the abnormalities of the EEG, there was a poor correlation with the other phenomena of the PSE syndrome. The bad results of the administration of lactulose was probably due to the mild grade of PSE and the low dosage.

Elkington (1969) (86) performed a carefully conducted double blind within-patient trial in 10 patients. The 10 patients studied had received previous

treatment with neomycin and protein restriction for encephalopathy which was clinically stable at the time of the trial. All had histologically proven cirrhosis. Of these 10 patients 7 were selected for the double blind trial during which they were kept on a constant restricted protein intake. Each patient was used as his own control and the trial was divided into 4 periods.

1. Control period;
2. Treatment with either lactulose or sorbitol at random for 6-27 days;
3. Control period of observation;
4. Treatment period with either sorbitol or lactulose, employing the drug which had not previously been administered.

Sorbitol 70 g/100 ml was presented in a syrup similar in flavour and colour as the lactulose syrup. The mental state was assessed and graded daily according to modified criteria of Parsons-Smith (87) and serial EEG's were interpreted "blindly" at the end of the study. Lactulose therapy resulted in improvement of 5 of the 7 patients studied. In all 5 stool pH and arterial ammonia concentration were reduced by lactulose. Improvement in the EEG occurred in 4. In the other 2 there was no decrease in stool pH or blood ammonia levels. Overt neurological signs of PSE had been present in only one of the seven patients at the time of study. This patient, when neomycin was replaced by sorbitol, relapsed into deep coma, but recovered by the repeat administration of neomycin. Lactulose therapy alone in this patient was associated with dramatic improvement in mental state and return to nearly normal blood ammonia levels and EEG. This improvement was maintained during long term lactulose therapy on a double protein intake. Lactulose was also administered on a long term basis (1-10 months) to seven patients. All showed satisfactory results enabling neomycin to be discontinued and protein intake to be doubled. Of the 62 serial stool specimens obtained from five patients, results indicate that here was no significant alteration in the recoveries of total aerobes or anaerobes. These observations suggested that lactulose may be useful in controlling chronic PSE.

Conn (88) performed in 1977 a randomized double blind clinical trial in which

he compared neomycin and lactulose in 33 cirrhotic patients with chronic portal systemic encephalopathy. Patients selected for the study were cirrhotic patients with or without portacaval anastomosis who had developed chronic or recurrent PSE. At the time patients entered the protocol, it was required that they were stable, were not azotemic, did not have intercurrent infections and did not receive encephalo-pathogenic medication. Patients remained in the hospital throughout the study. Each patient served as his own control. Because two different drugs were used, a double drug system was devised. Lactulose was paired with placebo tablets identical to neomycin tablets. Neomycin was administered along with sorbitol. Sorbitol however was not a placebo. Another drug like glucose would not have been possible since it would be immediately identified by the absence of cathartic activity. After an initial period of stabilization, control period I was begun. When patients remained well, dietary protein was gradually increased by 5 to 10 g/day and the control period was extended. During treatment A, which continued for at least 10 days the patients received either sorbitol-neomycin or lactulose placebo. Then control period II was started, which lasted for 10 days or more and was followed by treatment period B, during which lactulose-placebo was given. Then control period III was started. The medications were administered randomly by a sealed envelope system. Neomycin was administered in a dosage of 1.5 g/day and the liquids at 25 ml a day. This dosage was adjusted to induce 3 or more bowel movements. The pH of each stool specimen was measured and dietary protein was kept at a constant level throughout the study. Mental state was assessed at least 3 times daily. A trailmaking test was done and asterixis was recorded. EEG's were performed twice weekly. A PSE index was used as an arbitrary index of therapeutic efficacy. This index was based on the degree of abnormality of each of the various parameters measured e.g. mental state, trailmaking time, EEG, asterixis and arterial ammonia concentration. Thirty three patients satisfied the criteria for inclusion in the study. Twenty nine had alcoholic cirrhosis and 4 had a post-necrotic liver. A porta-caval shunt was performed in 15 of these 33 patients. 18 patients were

randomly selected to receive lactulose and 15 neomycin-sorbitol as treatment A. Patients who completed treatment A were then given the other drug.

The authors claimed that lactulose was considered effective in 15 of 18 patients, whereas neomycin-sorbitol was effective in 13 of 15 patients. In the cross-over study, lactulose was considered effective in 26 of 29 patients compared to 24 of 29 with neomycin-sorbitol. Mental state and asterixis were significantly improved by neomycin-sorbitol and by lactulose and deteriorated to pretherapy levels when these treatments were discontinued. EEG recordings improved to an equal degree with lactulose and neomycin-sorbitol. Improvement in trailmaking test time was observed with both neomycin-sorbitol and lactulose, but the differences were not significant. Mean ammonia concentrations were similar after both forms of therapy. In the cross-over comparison of neomycin-sorbitol and lactulose each of the indices of PSE was analyzed. Both neomycin-sorbitol and lactulose however showed no significant difference of improvement in asterixis and mental state. In the trailmaking test, time to perform the test was significantly reduced by lactulose but not with neomycin and sorbitol. Arterial ammonia levels were reduced by both neomycin and lactulose. The mean grade of EEG abnormality improved significantly with both lactulose and neomycin. The PSE index showed improvement in all patients who received lactulose. Of the 15 patients treated with neomycin-sorbitol 13 improved. In the cross-over study the PSE index was greater for lactulose than for neomycin-sorbitol. Both therapies reduced stool pH. The authors concluded that both lactulose and neomycin-sorbitol are effective in the treatment of chronic portal systemic encephalopathy.

Atterbury (1978) (89) reported a double-blind randomized study in which he compared the efficacy of lactulose with neomycin-sorbitol in 15 episodes of acute nitrogenous portal-sytemic encephalopathy. All the patients had underlying cirrhosis. Twenty episodes of acute PSE in ten patients who had suddenly developed acute PSE during the therapy were included in the series. In this study chronic PSE was defined as the state in which recurrent or continuous encephalopathy develops. Acute PSE was defined as the sudden develop-

ment in a cirrhotic patient of encephalopathy precipitated by nitrogenous substances in the absence of other explanations for encephalopathy. Patients were randomly assigned by sealed envelope technique to receive neomycin-sorbitol or lactulose. Patients selected to receive neomycin-sorbitol (1.5 g neomycin and 50 ml syrup every 1-2 hrs until two bowel movements had been passed). Patients receiving lactulose received 50 ml every 1-2 hrs until also two bowel movements had been passed. Dietary protein was kept constant at a level of 10-20 g daily as determined by the house staff. On the initial day of therapy both groups received a cleansing tapwater enema.

Several parameters were used to evaluate improvement and were brought into a PSE index: mental state, NCT (Number Connection Test which is the time in seconds it takes the patient to perform a simple psychometric test), EEG and arterial ammonia concentration. Patients were continued on the regimen to which they were randomized until maximum clinical response had been achieved. Lactulose and neomycin-sorbitol were equally effective in the treatment of acute PSE in this investigation. Both therapeutic programs were successful in more than 80% of the episodes. The improvement was in both groups demonstrated by a similar reduction of the PSE index. The only difference in results induced by lactulose versus neomycin-sorbitol was in the degree of reduction in stool pH: lactulose reduced the pH more than neomycin-sorbitol. The author concluded that lactulose is an effective alternative to neomycin in the treatment of acute PSE as well as chronic PSE (89).

1.4.4. Lactulose in acute portal-systemic encephalopathy

The efficacy of lactulose in the treatment of portal-systemic encephalopathy has been reported by a great number of investigators since it was first used. Although lactulose has primarily been studied in patients with the chronic recurrent form of the disease, a few papers have been published specifically about the treatment of patients with acute PSE. DeMeulenaere (60) reported in 1969 the use of lactulose in five patients with acute PSE (see chapter

1.4.1.). He concluded that lactulose was useful in acute PSE. The good clinical response was accompanied by a decrease in venous ammonia levels.

Fung (75) (see chapter 1.4.2.) reported eleven cases with evidence of acute hepatic encephalopathy treated with lactulose. There was an improvement of 54.5% directly attributed to lactulose.

Simmons (82) (see chapter 1.4.3.) performed a controlled investigation of lactulose in the treatment of PSE. Eleven patients received 80 g of lactulose daily while 10 patients received 60 g of glucose as control. Patients receiving lactulose showed significant reduction in both encephalopathy and blood ammonia levels.

Cassi reported a study in which he succesfully treated 5 patients with acute PSE (90).

Fessel (91) reported a controlled study in which lactulose was administered in 24 episodes of acute nitrogenous hepatic coma. The results were compared with 24 retrospectively matched episodes of encephalopathy treated with neomycin. The two groups were similar in type. Lactulose effectively reversed coma in 20 of the 24 episodes. Neomycin was effective in 23 of the 24 episodes. Improvement of arterial ammonia, EEG and other measures of encephalopathy were similar in the two groups. The study demonstrated that lactulose was effective in the treatment of acute PSE.

Atterbury (89) (see chapter 1.4.3.) compared in a double-blind randomized study the efficacy of lactulose with neomycin/sorbitol in 45 episodes of acute PSE. Two thirds of the patients in each group returned to normal mental status and more than 80% in each group showed at least one grade improvement in mental state. The only difference in results between those two groups was a reduction in pH of the stools in the lactulose group. These data suggest that neomycin/sorbitol and lactulose are equally effective in the treatment of acute PSE.

1.4.5. Lactulose enemas

Lactulose is degraded by bacterial enzymes in the intestine. When administered orally it is not degraded until it reaches the lower intestine 1-8 hours later and the beneficial clinical effects of lactulose are seen within 24 to 48 hours. It therefore seemed reasonable that if lactulose was injected directly into the colon by enema the clinical response might be achieved more rapidly. The first report of rectal enemas of lactulose in the treatment of PSE appeared in 1969. In an uncontrolled observation 300 ml of lactulose syrup (200 g and 700 ml water) was administered with a balloon catheter to assure retention of lactulose for at least 20 minutes. Mental state improved very rapidly and sometimes spectacularly. Also there was a marked fall in venous ammonia concentration and improvement in the EEG tracings (60).

Imler (see chapter 1.4.2.) (76) performed a study in patients with lactulose enemas. He reported no succes in their treatment.

Cassi (see chapter 1.4.4.) (90) performed a study in six patients with hepatic coma. One or 2 enema's containing 300 ml of lactulose were administered. Three of the patients awoke within a few hours. The others died.

Kersh (92) performed in 1973 a study in patients with hepatic encephalopathy in four patients in which he administered lactulose by retention enema. All patients showed dramatic improvement in clinical grade of coma within 12 hours. Arterial ammonia and cerebrospinal fluid ammonia levels showed a marked decline. The pH of the stool also decreased. They concluded that clinical and biochemical response occurred much more rapidly than in cases treated with oral lactulose.

Ratnaike (93) studied in 1975 six patients with severe liver failure. In these patients a colonic washout was performed, containing equal parts of lactulose and physiological saline (lactulose 50%). Two litres of the solution were administered to each subject by rectal tube over a one hour period. For the state of comparison, in two patients, the lactulose physiological saline washout was preceded by a "control" washout. In one patient the

control washout was performed using physiological saline pH 6.0 and in one patient acetate buffer at pH 4.5, the pH corresponding to that of the lactulose solution. Estimation of blood ammonia levels and EEG were performed before and after treatment. In five patients a significant fall was recorded in blood ammonia level as a result of lactulose therapy. In the sixth subject, an enema with buffered physiological saline with pH 4.5, induced a fall in ammonia, which was not increased by subsequent lactulose solution. Improvement in the EEG, was recorded in all periods where a lactulose colonic washout produced a fall in blood ammonia level. Van Waes (94) reported in 1979 a study with lactulose enemas during 34 episodes of acute PSE in 18 different patients. All patients had liver cirrhosis, 4 had a porta-caval shunt. Lactulose enemas consisted of lactulose syrup 300 ml diluted with tap water 300 ml. The pH of the solution was 3.85. Enemas containing 1000 ml of a 30% glucose solution in water (8 times) or tap water alone were used as control in 13 episodes. Before the enema, the degree of encephalopathy was evaluated clinically according to criteria of Parsons-Smith (87). Lactulose enemas decreased blood ammonia and reversed clinical and EEG manifestation of encephalopathy in 30 out of 34 episodes of acute portal-systemic encephalopathy in 18 patients with liver cirrhosis. The results became apparent within hours and were suggested to depend on effective acidification of the colon. The authors concluded that lactulose enemas are indicated for prompt control of acute episodes of PSE, when oral therapy is not sufficient to control hyperammonemia.

Conn (95) reported a study with seven patients, all treated with lactulose enemas. He administered one liter enemas that contained 500 ml of lactulose and 500 ml of water at 6-hour intervals. There was a good response in 10 of the 14 episodes and normal mental state was restored within 24 hours. In 3 patients mental state did not normalize. In all 3 patients, impressive reductions in arterial ammonia occurred.

Table I: Results of uncontrolled trials of lactulose in the treatment of hepatic coma

Table I

UNCONTROLLED TRIALS

Author	Year	Evaluated number	Daily dose	Duration	Comparison periods			Responding patients*				No. patients succesfully treated	Remarks
					protein*	neomycin	control	clin.	EEG	NH3	pH		
Markhoff	1966	1	30 g/dd	-	-	-	-	+++	-	-	-	1	
DePalma	1968	5	90-300 ml/dd	5-12 d	-	-	-	+++		down		5	
Lande + Clot	1968	12	100 ml/dd	5-10 d	-	-	-	+++	++	down	-	10	
Rottiers	1968	5 (A)	150 ml/dd	invar.	-	-	-	+++	++	down	-	5	
		6 (B)	150 ml/dd	invar.				+++		down		2	
Fung + Khoo	1968	2	30 ml/dd	-	70-80 g	-	-	+++	+++	-	-	2	
DeMeulenaere	1969	5A	150 ml/dd	3				+++	+++	down	-	5	
		7B	150 ml/dd	chronic	1.2 g/dd	-	-	-	-	down	-	6	
		2C	300 ml/dd rectal	1 d				+++	+++	down	-	2	
Helms	1969	10	20-200 ml/dd	-	-	-	-	-	-	-	-	2	
Combes	1969	6	100 ml/dd		up		sorbitol	+++				6	
Gauthier	1970	37	45-150 ml/dd					+++				27	
Geoffroy	1970	20	100 ml/dd		up			+++		down	-	18	
James	1971	6		10 d				+++				5	
Kardel	1972	11		4 w				+++	+++	--->		3	
Almici	1973	19	90-180 ml/dd	7 d				+++	+++	down		16	
Eisenburg	1974	14						++				10	
Reikowski	1974	36	45-135 ml/dd					+++				35	
Seidlovia	1974	14	96 ml/dd	2-12 m	up			+++		down		11	

*) up = protein intake increased; 0 = not possible to increase

*) ++ = moderate improvement; + = small change only; 0 = fluctuating
+++ = marked or virtually complete improvement; down = decrease; ---> = unaffected
C = were sorbitol, glucose or magnesium sulphate

Table II: Results of controlled trials of lactulose in the treatment of hepatic coma

Table II

CONTROLLED TRIALS

Author	Year	Evaluated number	Daily dose	Duration	Comparison periods protein*	neomycin	control	Responding patients* clin.	EEG	NH3	pH	No. patients succesfully treated	Remarks
Ma	1969	10	90 ml/dd	1-2 m	-	L=N	-	++-+++	+++	-	-	6	
Rorsman	1970	3	60-160 ml/dd	5-25 m	up	-	-	++-+++	-	-	-	3	
Siebner	1970	12	150 ml/dd									11	
Zeegen	1970	7	100-150 ml/dd	8-25 d	up		L>C			down	down	3	MgSo4 control
Fung	1971	5	90 ml/dd	13 m		L>N		+++				5	
Imler	1971	7	90 ml/dd	7 d		N>L		+++	+++	down		2	
Bircher	1966+1971	6	105 ml/dd		up	L=N	L>C	++-+++	++	down	down	6	Sorb + MgSo4 control
Rodgers	1973	6		1 year			L=C					3	Sorbitol
Orlandi	1981	91	30-105 ml/d	18.6 d		L>N		+++	+++	down	down	28	

CONTROLLED DOUBLE BLIND TRIALS

Simmons	1970	26	90-200 ml/dd	10 d	up	-	L>C	++	-	down	-	13	
Brown	1970+1971	7	45-180 ml/dd	1-8 m	up	-	L>C	++-+++	++	0	down	7	
Germain	1973	9	60 ml/dd	15 d	-	-	L=C	++	+++	0		5	
Elkington	1969	7	90-134 ml/dd	7-27 d	-	-	L>C	+++	++	down	down	5	
Conn	1977	29	125	11 d			L=C	+++	++	down	down	26	
Atterbury	1978	10	150 ml			L=N		+++			down	10	

*) up = protein intake increased; 0 = not possible to increase

*) ++ = moderate improvement; + = small change only; 0 = fluctuating
+++ = marked or virtually complete improvement; down = decrease; ---> = unaffected
C = were sorbitol, glucose or magnesium sulphate

I.5. Neomycin in hepatic encephalopathy

I.5.1. Neomycin: the mode of action and efficacy

Neomycin is a amino-glycoside antibiotic isolated from a strain of Streptomyces fradiae.

The drug is effective against most gram-negative and some gram-positive aerobes, but it has virtually no effect on the anaerobic flora (96,97).

Its activity is not due to a single substance but to a number of active substances entitled the neomycin complex (98). When Silen (99) used the drug in dogs, it became apparent that it significantly lowered the ammonia levels in the venous mesenteric blood, even when protein feeding was continued (100).

It soon became the drug of choice in the treatment of hepatic encephalopathy (101,102,100) and various investigators claimed beneficial effects (103,104). Its mode of action was claimed to be based on the fact that it reduces the faecal flora. The success of neomycin treatment confirmed the idea that ammonia originated solely from bacteria and that the decrease in plasma ammonia levels occurred due to the anti-microbial properties of neomycin. Dawson however, could not correlate the clinical improvement with bacteriological changes in the stools (101). He concluded also that neomycin certainly did not sterilize the stools and that several species of ammonia producing bacteria remained.

Also in the study of Sabbaj (105) no correlation was found between clinical improvement and bacteriological changes in the faeces. He discovered that although deaminating capacity was common among the faecal bacterial species oral treatment with neomycin or kanamycin caused no reduction in the numbers of deaminating bacterial species.

1.5.2. Uncontrolled and controlled trials

In 1954 Sherlock (106) introduced oral broad spectrum antibiotics and enema as a basis for treating hepatic coma. In 1955 Silen (99) demonstrated in dogs that ingested neomycin is highly effective in reducing ammonia levels. Fischer (100) reported in 1957 a study of 11 patients to whom neomycin was administered. They had hepatic cirrhosis due to excessive alcohol ingestion. Patients were divided up into two groups. The first group of 8 patients without neurologic manifestations was given a protein diet 110-120 g and received 12 g neomycin. The second group consisting of three patients with neurologic manifestations, was given 12 g of neomycin daily, after control blood ammonia determinations were obtained. Results indicate that the oral administration of neomycin resulted in a significant decrease in blood ammonia levels in patients with cirrhosis, regardless of the presence or absence of neurologic changes.

In 1957 then Dawson (107) reported a study of 12 patients with acute hepatic coma and 8 patients with chronic hepatic coma, who received neomycin for periods up to ten months. He observed the clinical state, blood ammonia levels and the effect on stool flora. The dosage was 4-10 g daily. Six chronic patients showed pronounced clinical benefit which was associated with a fall in the fasting arterial blood-ammonia level and an improvement in the EEG. One patient relapsed after ten months of treatment. The effect on the stool flora was variable and could not be correlated with the clinical benefit or with the fall in arterial blood ammonium level. Seven out of 12 patients with acute hepatic coma showed initial improvement, but other forms of treatment were used simultaneously.

In 1958 Summerskill (108) performed a controlled study with neomycin on six patients. A daily assessment of the neuropsychiatric state was carried out. In his study he compared neomycin with chlortetracylin and chloramphenicol. Case 1, 2 and 3 were of interest, because comparative studies were possible. Neomycin appeared to be more effective than chlortetracyclin, sigmamycin and

chloramphenicol. The results underline the importance of oral antibiotic therapy and show that neomycin is the most effective of recommended antibiotics.

In 1958 Fast (109) reported a study of eleven patients with liver disease and hepatic coma. Nine patients had cirrhosis associated with chronic alcoholism. One had cirrhosis of unknown etiology and one had viral hepatitis. Patients were divided into three groups. Group I consisted of two patients with episodic stupor who were studied under the controlled conditions of a metabolic ward. Group II consisted of six patients having progressive hepatic failure, as indicated by elevated serum bilirubin concentrations, the presence of ascites and the spontaneous appearance of hepatic coma. Group III consisted of three patients with hepatic coma in whom massive gastrointestinal hemorrhage was the precipitating factor. In group I neomycin was administered and dietary protein gradually increased from 30 to 90 g. No alteration in mental status was noted. Later paromycin and neomycin were given when coma was evident. When given in a dose of 4.0 g or more daily clinical improvement resulted within 48 hours. Complete recovery was seen within 4-5 days. In patient 2 severe impending coma occurred during an intake of 90 g of protein. A dramatic response occurred within 24 hours after administration of 4 g of paromycin. Arterial ammonia in patient 1 correlated with clinical response. Patients in group II and III were treated with enemata. Sedatives and other drugs known to cause hepatic coma and excess of dietary protein were omitted. Neomycin or paromycin were administered in daily doses of 1 g or more four times a day. In group II only one patient recovered completely. In group III all three responded to therapy and two recovered completely. One died following recurrent haemorrhage. The authors concluded that neomycin and paromycin orally used were effective therapeutic agents in hepatic coma. In case of hepatic failure associated with hepatic coma antibiotics are recommended, allowing the consumption of normal amounts of protein.

Stormont (110) carried out a study in 1958 in 68 patients and observed 70 episodes of hepatic coma. He used Summerskill's criteria for selection of

patients. Protein was completely eliminated from the patients' diet. Nitrogenous material in the colon was removed initially by enemas. Neomycin or paromycin was administered orally in doses of 4-8 g daily. Chlortetracyclin 2 g daily was given to 12 patients. Protein was reinstituted in about 30 g amounts on the second to fourth day. When improvement occurred, dietary protein intake was gradually increased to 60 g daily, while antibiotics were maintained. If progress continued, antibiotics were gradually decreased and discontinued. Recovery was assumed to mean improvement sufficient for the patient to leave the hospital. Of the 58 episodes (excluding the 12 patients receiving chlortetracylin) 34 were treated with paromycin which resulted in 14 recoveries. 24 were treated with neomycin resulting in 9 recoveries. Thus, there was essentially no difference between the various antibiotics used in conjunction with readministration of protein. Comparison with 35 patients treated with complete protein withdrawal and chlortetracyclin also revealed no important difference in improvement and recovery. Stormont concluded that neomycin and paromycin in the treatment of hepatic coma was found equal, if not superior to therapy with chlortetracylin.

Faloon (104) studied 22 patients with a history and physical findings consistent with alcoholic cirrhosis during 25 episodes of coma. The usual regimen of therapy in these patients included administration of 20 to 40 g of protein and oral neomycin 12 g daily. Recovery was assumed to have occurred when patients were in a stable alert state and could be discharged from the hospital. Of the 22 patients receiving neomycin 11 died but 9 improved prior to death. Of the 25 episodes of coma, recovery was noted in 14 instances. Of the 18 patients receiving protein from the beginning of therapy together with neomycin, seven lived and eleven died, but improvement was noted prior to death in nine of the latter. A decrease in blood ammonia during therapy was observed in 16 of the 18 patients in whom it was measured.

In 1981 Uribe (111) carried out a randomized double blind comparison of lactose enemas plus placebo tablets versus starch enemas plus neomycin tablets in 18 patients having acute portal systemic encephalopathy. Acute PSE

was arbitrarily defined as the sudden development of encephalopathy in a cirrhotic patient. Before inclusion in the study patients must have developed an acute episode of PSE within 24 h. Eight patients received a one liter lactose enema of 20%. Simultaneously they received placebo tablets identical to neomycin tablets. Ten patients received 1 l starch enemas (10%) plus two 1.5 g neomycin tablets. The following parameters were assessed: mental state, asterixis, EEG, blood ammonia concentration. These parameters were computed in a PSE index. Clinical and biochemical improvement was observed after lactose enemas in 7 of 8 patients and in 7 of 10 patients treated with starch. Both treatments significantly decreased the frequency of asterixis and the level of blood ammonia, and improved EEG. Although this double blind trial was done to demonstrate the effect of lactose, neomycin proved to be an efficacious therapeutic agent for hepatic coma. One should keep in mind however that the beneficial effect of both treatments to a significant extent could have been the consequence of the cleansing effect of both enemas.

In a double blind trial Hirayama (1982) (112) studied 24 patients with advanced chronic liver disease who received 1.2 g of nicotine hydroxamic acid daily (N.H.A.) and 23 patients who received 1.5 g of neomycin. Patients receiving N.H.A. showed significant improvement in blood ammonia levels as compared to patients receiving neomycin.

Conclusions

Several studies have been devised in order to study the effect of lactulose on hepatic coma. The early studies however were not controlled and the data concerning clinical response to therapy were insufficient. However of the 16 uncontrolled trials (table I) in which 217 patients were studied 170 responded well to lactulose treatment (79%). After 1969 studies became more controlled. A few of these studies compared lactulose with other therapeutic agents like neomycin, broad spectrum antibiotics and laxatives like sorbitol and $MgSO_4$. One of these studies performed in 1966 and 1971 by Bircher (77,78) under controlled conditions was of excellent set up and carefully designed.

Of the 56 patients in these studies 39 (69%) responded well to therapy. a few truly objective double blind studies on the effect of lactulos hepatic coma, have been conducted (table II). Of these studies, the lar was published by Simmons and Conn. Of 179 patients entering the tria (52.5%) responded well to therapy, which is almost 30% less than the out of the early studies. It appears as if the better the trial is control the worse the result is of lactulose treatment of hepatic coma. Althougl last controlled studies were double blind a control group using placebc not included, most likely for ethical reasons. As an outcome of sev studies in which neomycin was administered orally and the ammonia b levels decreased, thereby ameloriating the PSE, neomycin was chosen treatment of hepatic coma. Very few controlled studies on neomycin treat have been carried out. Atterbury (89) compared neomycin - sorbitol lactulose and found the drugs equally effective. Neomycin however was together with sorbitol and Brown (83) proved that sorbitol is a very alternative for lactulose. Conn (88) carried out a comparable study using neomycin in combination with sorbitol. Orlandi (80) recently condu a controlled study on neomycin therapy. He demonstrated a similar cours the syndrome when patients with grade I hepatic encephalopathy were tre with neomycin and MgSO4 or with lactulose. Results were better with neom in severe hepatic encephalopathy. A control group receiving placebo was included however and the study was not double blind. Although both drugs used worldwide in the treatment of hepatic coma, no double blind contrc study with a placebo group has ever been performed.

1.5.3. Lactulose and neomycin combined in the treatment of hepatic coma

Both drugs are well accepted in the treatment of hepatic coma and the me nism of action is based on the presumed toxic effect of ammonia and on essentially intestinal origin of circulating ammonia. According to literature neomycin is known to exert its effect by destroying the aer

flora and lactulose needs bacteria to break up into several organic acids which lower the intestinal pH and exert a laxative effect due to their osmotic property and to incorporate ammonia into amino acids. Lactulose and neomycin alone have been found to be equally effective in the management of PSE (see chapter 1.4) but neomycin is not the first choice because of its side effects (renal toxicity, malabsorption, inner ear deafness). Theoretically there is no need and no expected benefit of using these drugs together. One has suggested that neomycin does not kill all the laxative fermenting bacteria, and therefore that the drugs could potentiate each other. Because of the possible superimposed effects of both drugs Pirotte (113) performed a study in 1974 on nine cirrhotics with chronic PSE. In each patient the study was carried out in three successive periods.

Period 1: First patients were given high doses of lactulose. Three of the subjects also received 2.5 g neomycin daily orally. The treatments soon brought an improvement in the clinical and biological state.

Period 2: Stabilization period.

Period 3: During this period, the patients had complete rest and were kept on a constant diet, identical to that of period 2. Each patient received 15 g lactulose 3 times a day, then no drug treatment for 5 days. During the next 10 days each patient was given 250 mg neomycin 4 times a day. One day later 45 g of lactulose was added to neomycin and this double treatment was continued for 12 days. Pirotte concluded that, used separately, neomycin and lactulose significantly reduce the basal ammonemia to values which were not statistically different from each other, but the association of both drugs lowered the basal ammonemia to values significantly lower than those recorded when the drugs were given alone.

Imler (76) (see chapter 1.4.2.) found in two patients, in whom neither lactulose or neomycin completely restored the mental state, EEG or blood ammonia levels, that the combination of both drugs did restore these measures.

Conn (95) performed a study in 22 cirrhotics and studied the stool pH of patients receiving the two agents individually and in combination. Based on

the changes in stool pH it appeared that neomycin has no effect on acidity in one third, partially in the other third but completely inhibits the acidification in the last. He suggested that one might expect optimal action of both agents in about one-third of the number of patients. Hoyumpa (114) suggested in his study that the lactulose splitting bacteria appear to be relatively resistant to neomycin which is why a combination could work in some cases. As all the authors seem to agree: it is not yet clear whether lactulose should be used alone or in combination with neomycin.

I.6. Ammonia in relation to the hepatic coma syndrome

There is considerable clinical and experimental evidence that abnormalities in ammonia metabolism are somehow involved in the pathogenesis of hepatic coma (115,116) although a poor correlation between the stage of hepatic encephalopathy and ammonia concentration in blood has also been reported (117,118,119). Reasons for considering ammonia as an important agent in the genesis of hepatic encephalopathy are:

A. Lactulose and some other cathartics, neomycin and some other antibiotics, protein restriction, colon exclusion and other therapeutic measures appear to be of benefit in hepatic coma and all reduce ammonia levels in blood.

B. A great number of investigators have reported a good correlation between plasma ammonia levels and the degree of hepatic encephalopathy (120,121).

C. A form of coma can be induced in patients with chronic failure after ammonia loading (122).

Ammonia is generated in the gastrointestinal tract from bacterial and non-bacterial sources (17,26) and in liver, muscle, brain, kidney and erythrocytes via the purine nucleotide cycle (123).

Ammonia production by muscle only occurs after heavy excercise, but in 10% of the patients with liver disease, muscle has been reported to increase arterial ammonia (124). Ammonia in blood, largely derived from the gut is meta-

bolized in the liver to urea. The urea then is excreted by the kidney and can be hydrolysed to a certain degree in the gut. When liver function is impaired, or when portal blood is shunted around the liver into the systemic circulation, muscle may fullfill an important role in homeostasis. In hepatic disease with hyperammonemia, skeletal muscle is able to metabolize part of the arterial ammonia supply (125,126). Ganda (127) claims that the ammonia taken up by muscle is released in the circulation as glutamine which than acts as a non-toxic nitrogen carrier. Imler however suggests that the glutamine release by muscle is not sufficient to explain the total ammonia uptake by muscle (128). Therefore high glutamine content of plasma together with a hypoalanemia in patients with liver disease has been suggested to result not only from the metabolic processes in muscle, but also from processes occurring in kidney, gastrointestinal tract and liver (129).

Ammonia is still present in many hypotheses concerning the genesis of hepatic encephalopathy.

1. Ammonia can freely enter the brain.

   Inside the brain it is thought to be metabolized via the alpha-keto-glutarate-glutamate sequence. As a result depletion may occur of alpha-ketoglutarate which may result in a decreased operation of the cycle. This results in a fall of high energy phosphate and oxygen consumption. Studies in dogs did show a reduction in many of the tricarboxylic acid cycle substrates intermediates, although results did not differ from a simple sedative or anaesthesia (130). These changes may be secondary rather than primary to coma. In an acute hepatic coma rat model, when rapidly freezing the brain, no major changes occurred in energy substrates (131,132).

   Several other hypotheses of ammonia in relation to the hepatic coma syndrome have been mentioned.

2. An acute toxic effect of ammonia on rat brain metabolism has been suggested. Hawkins used the new-freeze blowing technique, which permits freezing of supratentorial brain in rats within 1 sec. and without anaesthe-

sia (133). His study showed no change in brain high energy phosphates ir central cortex and hemispheres and therefore suggested that ammonia toxicity was related, not to a disturbance of brain energy balance but to a direct neuronal effect of ammonia on the brain cells. This suggestion was also put forward in other publications (134) but is difficult to study in vivo.

3. In 1977 Zieve (130) postulated the synergism between mercaptans, ammonia and short chain fatty acids in the production of coma. Ammonia toxicity was enhanced three to four fold by the addition of methanethiol. Similarly, the coma dose of sodium octanoate in the presence of methanethiol was one third the dose required to induce coma by the short chain fatty acid alone.

4. The latest concept, which brings the ammonia together with the amino acid neurotransmitter hypothesis of Fischer, has been published by James (135). In his hypothesis he claims that hyperammonemia raises the concentration of neutral amino acids in the brain resulting in changes in neurotransmitter metabolism. Since ammonia stimulates brain-glutamine synthesis, increased outward transport of glutamine occurs at the expense of exchange for plasma neutral amino acids. Because the plasma neutral amino acid profile is distorted in liver disease, increased transport of neutral amino acids over the blood-brain barrier also results in a distorted pattern in the brain, especially including increased levels of aromatic amino acids. Because these aromatic amino acids are precursors of neurotransmitters, the whole sequence may then result in a distorted neurotransmitter pattern and encephalopathy.

5. For the sake of completeness, the GABA theory should be mentioned which is the only theory, which does not include ammonia. GABA is an inhibitory neurotransmitter, which is said to be formed by the anaerobic bacterial action (136) but can probably also be formed in the mucosacel of the intestine. It is degraded in the liver. In acute fulminant hepatic failure and encephalopathy, gut-derived GABA in plasma crosses an abnor-

mally permeable blood-brain barrier and by mediating neural inhibition is claimed to contribute to hepatic encephalopathy. An increased number of GABA receptors in the brain found in liverfailure would then increase the sensitivity of the brain to GABA-ergic neural inhibition.

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Chapter II

IN VITRO AMMONIA AND AMINO-ACID PRODUCTION IN SMALL AND LARGE INTESTINE OF THE RAT AND THE INFLUENCE OF NEOMYCIN AND LACTULOSE

II.1. Introduction

Ammonia is claimed to contribute to hepatic encephalopathy. The presumptive etiological role has served as the base of many therapeutic measures. Most ammonia in the body is claimed to originate in the gut as a result of degradation of urea, protein and amino acids by bacteria (1,2,3). Urea is considered to be the main source of ammonia because large amounts (± 7 g/day) are degraded in the body (1), apparently as a result of the activity of the intestinal flora. Doubts about the contribution of urea to colonic ammonia production have risen however because the human colonic mucosa is impermeable to urea (4) and only small amounts of urea reach the colon from the ileum (5). Studies by Wrong (6) also show that endogenous urea is not, as been generally assumed, the major source of faecal ammonia. They confirm earlier studies that faecal ammonia and total nitrogen is in close equilibrium. Recent studies suggest that an important site of ammonia production must be the intestinal wall itself because germ-free dogs became hyperammonaemic after porta-caval shunt (7).

Matsutaka (1973) (8) discovered in rats that this gut ammonia production was glutamine dependent. He suggested that the nitrogen of glutamine and other amino acids catabolized were converted into alanine and ammonia. This production of ammonia had to be non-bacterial since he studied germ-free rats and as much ammonia and alanine accumulated in the perfusate as in experiments with conventional rats.

Windmuller and Spaeth (1974) (9,10) have identified the small intestine as

the site of this glutamine utilization where glutamine contributes significantly to the respiratory fuel of the intestinal mucosa. Weber (11) quantitated the ammonia released into the portal vein in dogs and demonstrated that the production of ammonia by jejunum and ileum was of equal magnitude as in the colon. This production could be accounted for by uptake of glutamine from arterial blood.

One of the therapeutic measures to reduce ammonia levels is the administration of neomycin and lactulose. Explanations, that the effects of neomycin and lactulose are achieved by their action on bacterial colon flora are conflicting. Neomycin lowers ammonia levels in the portal vein presumably by elimination of the aerobic flora (12). Such an effect of neomycin on ammonia levels is difficult to understand since the aerobic flora represents only a small fraction of the total flora in the intestine. Moreover, clinical state and blood ammonia do not always correlate with the observed alterations in flora induced by neomycin (13). Lactulose has been claimed to influence ammonia metabolism and absorption in the colon by a decrease of the luminal pH (14), leading to an osmotic diarrhoea (15) and provision of a carbon and energy source to assimilate ammonia by bacteria (16). However the mechanism by which lactulose might provide assimilation of ammonia by bacteria was not really elucidated. Whether lactulose and neomycin act directly on non-bacterial ammonia metabolism has never been subject to study. Little evidence exists to suggest that there are sources of gut ammonia production not attributable to bacterial metabolism within the colon. These considerations prompted us to study amino-acid and ammonia metabolism in the small and large intestine with particular interest for the metabolism of glutamine, alanine, ammonia and glutamic acid, and the effect of lactulose and neomycin. As a first approach to this problem, in vitro studies were carried out with fragments of small and large intestine. The production of ammonia was quantitated after addition of glutamine to the medium, and the influence of neomycin and lactulose on this ammonia production was studied.

II.2. Material and Methods

Rats.

Specified pathogen free (SPF) rats were obtained from the centralized experimental animal facilities of the University of Limburg.
Male Wistar rats were used weighing 250-300 gr. Rats were kept at 21°C and allowed free access to water and rat chow (formula SRM A120 Hope Farms, Woerden). Fasted rats were deprived of food for 24 hours before operation. Operations were performed between 8.00 am and 10.00 am.

Reagents.

Enzymes: Crystalline glutaminase (L-Glutamine-Amidohydrolase EC. 3.5.1.2.) was a product of Sigma U.S.A. The following were products of Boehringer Mannheim, Mannheim: Glutamate dehydrogenase [L-glutamate: NAD(P) oxidoreductase (deaminating), EC. 1.4.1.3], type II in 50% glycerol from beef liver (specific activity 120 U/mg); L-alanine dehydrogenase (L-alanine: NAD oxidoreductase) deaminating EC. 1.4.1.1. (specific activity 30 U/mg). Lactulose (Duphalac) was obtained from Duphar (Weesp, the Netherlands) and contained 667 mg lactulose/ml. Neomycin sulphate was obtained from Lundbeck (Amsterdam, The Netherlands).

Incubation procedures.

Through an abdominal incision the small and large intestine were removed. Small and large intestine were rinsed with icecold saline to remove luminal contents and opened. Longitudinal sections of gut approximately 1 cm in length were obtained weighing 35-40 mg wet weight. The intestinal pieces, cut along the mesenteric border were transferred to Erlenmeyer flasks containing 2 ml of icecold Krebs-Ringer buffer.
Tissue was then transferred to a fresh medium (38.5°) with and without glutamine (10 mM), and with and without lactulose or neomycin, incubated for 90 min in a shaking water bath at 60 cycles per minute (Grants Instruments,

London) and kept under carbogen atmosphere. After incubation, the tissue was frozen in liquid nitrogen and homogenized with a tissue grinder (size 0022 Firma Kontes van Oortmersen, The Netherlands). Medium and homogenates were deproteinized with perchloracid.

Determination of metabolites.

Ammonia was determined according to the method of Da Fonseca-Wollheim (17). Alanine, glutamine and glutamic acid were measured in the incubation media and tissue homogenates by an enzymatic micromethod using a centrifugal analyser (Cobas Bio, Hoffman La Roche). We followed the method of Williamson (18) for alanine, of E. Bernt (19) for glutamic acid and of E. Lund (20) for glutamine.

Lactate dehydrogenase was determined in the medium after centrifugation at 7000 x g for 2 min in an Eppendorf microfuge. Total lactate dehydrogenase (LDH) content of the tissue was measured after complete lysis, which could be obtained by homogenization of the tissue with a Ultra-turrax, 3 times for 7 sec on ice and subsequently by sonification for 40 sec, with intervals for 5 sec, amplitude 12 microns (Sonicator type ultrasonic desintegrator 100 watt model, NV Beun - de Ronde - HVL). Lactate dehydrogenase was measured with a Boehringer test kit based on the method of Wroblewski and La Due (23).

Interassay variations.

In order to standardize our amino acid determinations a standard curve was prepared in the same way as our tissue and medium and frozen at -70°. Four standards were measured together with all samples in order to correct all our measurements for interassay variations. Interassay variations of the amino acid measurements were obtained by measuring four standard aliquots on the same day under the same conditions as the samples, during the whole experiment.

- Alanine. The following standard concentrations were used: 16.5 - 33.0 - 82.7 and 165 µmol/L (n=15). The observed results were respectively 16.4 -

33.0 - 82.9 and 164.9 µmol/L.

Coefficients of variation (CV), expressed as a percentage of the mean were respectively 3.1% - 4.7% - 2.9% and 3.5%.

- Glutamic acid. The following standard concentrations were used: 16.6 - 32.3 - 65.0 - 131.3 and 250 µmol/L (n=8). The observed results were respectively 18.3 - 31.6 - 63.0 - 130.0 and 250.0 µmol/L.

  CV: 9.4% - 2.2% - 1.4% - 1.0% and 0.7%.

- Ammonia. The following standard concentrations were used: 50.0 - 100.0 - 200.0 and 300.0 µmol/L (n=20). The observed results were respectively 51.5 - 102 - 200 and 301 µmol/L.

  CV: 6.5% - 4.4% - 2.9% and 1.8%.

Intra-assay variations.

Intra-assay variations of the amino acid measurements were obtained by measuring 10 aliquots of these standards on the same day, under the same conditions. This procedure was repeated on three different days.

The intra-assay variations of alanine, glutamic acid and ammonia are respectively 2.7% - 0.5% and 0.9%. The minimum detection level of each alanine, glutamic acid, glutamine and ammonia in medium and tissue is 0.5 nmol/mg tissue.

The mean analytical recovery of alanine, glutamic acid and ammonia after 30 min and 60 min incubations with and without glutamine 10mM and with and without lactulose and neomycin were for alanine, glutamic acid and ammonia respectively 100% - 99.5% and 99.8%.

II.3. Results

Linearity in time and weight.

In order to test the linearity of the production of ammonia in the test system, incubations were performed during increasing time periods with

and without addition of glutamine. In fig. 1 the results of small and large intestine respectively are represented.

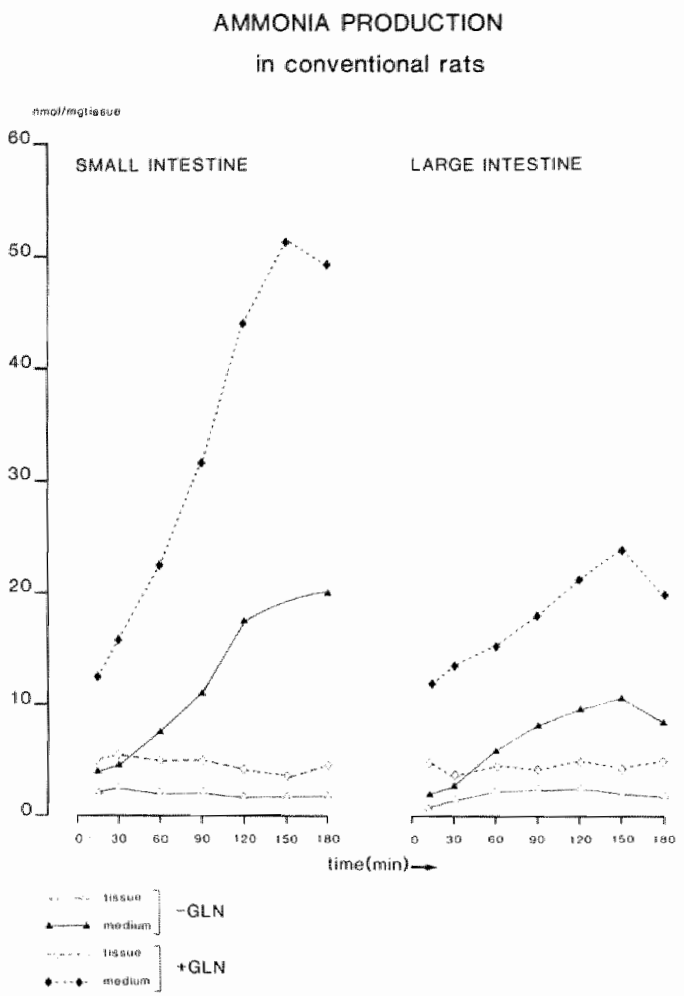

Figure 1: Ammonia production in the small and large intestine as measured in incubation medium and tissue after different times of incubation intervals (0 - 180 min.).
Incubations were performed with and without glutamine (10mM). Results are the mean of the triplicates and expressed in nmol/mg tissue wet weight$_{W.W.}$.

Production of ammonia in the incubation medium was linear until 150 min, thereafter production of ammonia declined suggesting that viability of tissue pieces decreased. The concentration of ammonia in tissue homogenates did not

increase substantially. To test the possibility that diffusion of O_2 and substrate might be rate limiting for ammonia production, segments of bowel were incubated with weights increasing from 20-200 mg (fig. 2).

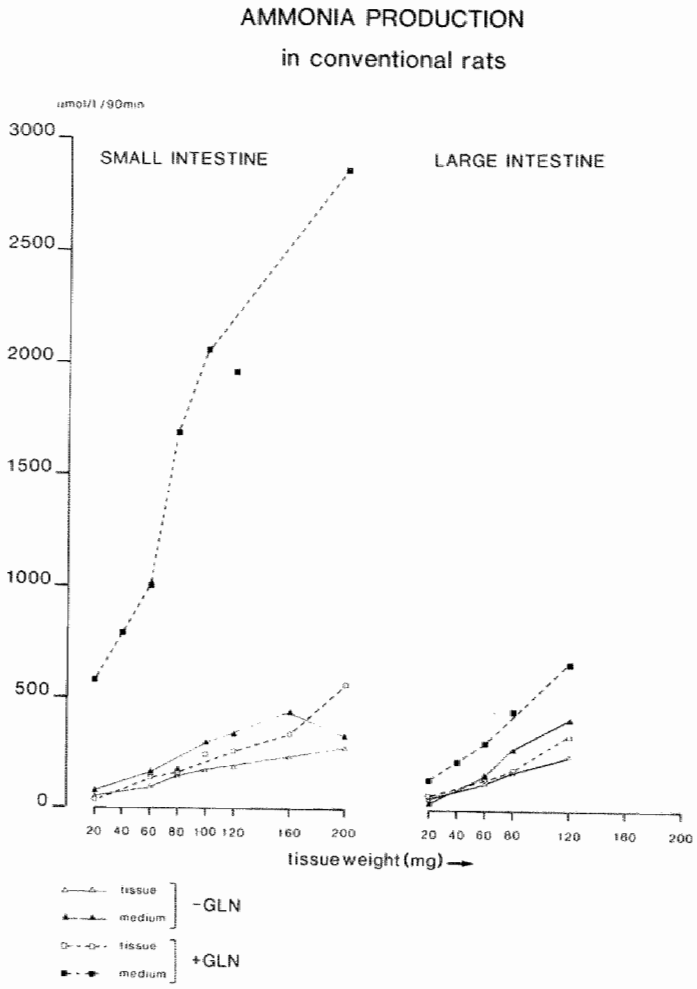

Figure 2: Ammonia production in slices of small and large intestine of different weight after 90 min. of incubation. Ammonia was measured in tissue and medium. The tissues were incubated in media with and without glutamine (10mM). Results are the mean of the triplicates and expressed in µmol/L/90min.

Ammonia production proved to be proportional to weight under these circumstances.

Ammonia and amino-acid release by small and large intestine and the influence of feeding and fasting.

Small and large intestine both from fasted and fed rats were capable to generate significant amounts of ammonia, alanine and glutamate in vitro in the presence and absence of glutamine (tabel I and II).

Table I

	Ammonia	Alanine	Glutamine	Glutamate
	Small intestine (fasted state - glutamine)			
Medium	10.19 ± 1.03	10.40 ± 1.0	< 2.5	5.19 ± 0.84
Tissue	2.13 ± 0.38	2.42 ± 0.24	< 2.5	1.55 ± 0.09
	Small intestine (fasted state + glutamine 10mM)			
Medium	35.40 ± 3.76	24.70 ± 1.25	448.30 ± 19.9	12.56 ± 2.53
Tissue	4.21 ± 0.15	2.75 ± 0.22	14.28 ± 2.0	2.68 ± 0.22
	Large intestine (fasted state - glutamine)			
Medium	3.84 ± 0.42	2.47 ± 0.3	< 2.5	1.47 ± 0.14
Tissue	2.58 ± 0.10	1.27 ± 0.4	< 2.5	1.76 ± 0.20
	Large intestine (fasted state + glutamine 10mM)			
Medium	15.20 ± 1.07	6.94 ± 0.5	448.3 ± 19.2	6.26 ± 1.07
Tissue	5.35 ± 0.53	1.48 ± 0.16	16.9 ± 2.22	3.15 ± 0.35

Table I: Ammonia, alanine, glutamate and glutamine concentrations in small and large intestine (fasted state) of conventional rats after incubations (90 min.). The mean of the concentrations were measured in tissue and medium (n=7, mean ± SEM). Glutamine in the incubation medium was 10mM. Results are expressed in nmol/mg $tissue_{W.W.}$/90min.

Table II

	Ammonia	Alanine	Glutamine	Glutamate
	Small intestine (fed state - glutamine)			
Medium	4.71 ± 0.62	7.53 ± 0.81	< 2.5	3.53 ± 0.30
Tissue	4.52 ± 0.53	6.76 ± 0.50	< 2.5	2.80 ± 0.43
	Small intestine (fed state + glutamine, 10 mM)			
Medium	32.04 ± 2.61	27.20 ± 1.75	417.0 ± 18.9	12.27 ± 0.75
Tissue	5.86 ± 0.42	8.15 ± 0.70	19.8 ± 5.8	4.00 ± 0.28
	Large intestine (fed state - glutamine)			
Medium	3.64 ± 0.74	2.00 ± 0.4	< 2.5	1.04 ± 0.18
Tissue	5.16 ± 0.43	3.95 ± 0.4	< 2.5	2.80 ± 0.32
	Large intestine (fed state + glutamine 10mM)			
Medium	10.30 ± 0.5	3.50 ± 0.4	461.0 ± 10.6	11.70 ± 0.6
Tissue	4.44 ± 0.25	3.95 ± 0.31	14.1 ± 2.3	2.50 ± 0.7

Table II: Ammonia, alanine, glutamate and glutamine concentrations in small and large intestine (fed state) of conventional rats after incubations (90 min.). The mean of the concentrations were measured in tissue and medium (n=7, mean ± SEM). Glutamine in the incubation medium was 10 mM. Results are expressed in nmol/mg $tissue_{W.W.}$/-90min.

In the fed state, without addition of glutamine, small intestine produces an equal amount of ammonia per unit weight when compared to the colon (table II). More alanine and glutamate was released by the small intestine than by the colon. Addition of glutamine (10 mM) increased the production of ammonia, alanine, glutamate in small and large intestine. More alanine, glutamate and ammonia was released by the small intestinal segments than by the colon segments.

Ammonia, glutamate and alanine were produced by small and large intestine in amounts which accounted roughly for the decrease in glutamine. No significant differences were noted when pieces of intestine were obtained from fed or fasted rats.

In order to estimate the damage to the fragments of bowel after 90 minutes of incubation upon exposure to lactulose, lactate was determined in tissue homogenates and proved not to increase when lactulose was added. In addition lactate dehydrogenase (LDH) release during incubation was determined and expressed as percentage of total cellular LDH content. It can be seen in table III that there is a small leakage of LDH into the medium during 60 min of incubation.

Table III

Conventional rat			
Small intestine	15.6 ± 2.6 %	Large Intestine	5.7 ± 1.1 %

Table III: Lactate dehydrogenase (LDH) measurements in the medium of small and large intestine. After 60 min of incubation expressed as percentage of the total LDH contents of the tissue prior to incubation (n=3, mean ± SEM).

The effect of lactulose on ammonia production in vitro.

Incubations were performed with lactulose in the medium in concentration ranging from 5-25% (W/V). Ammonia production by the small intestine decreased significantly when lactulose was added to the medium (fig. 3).

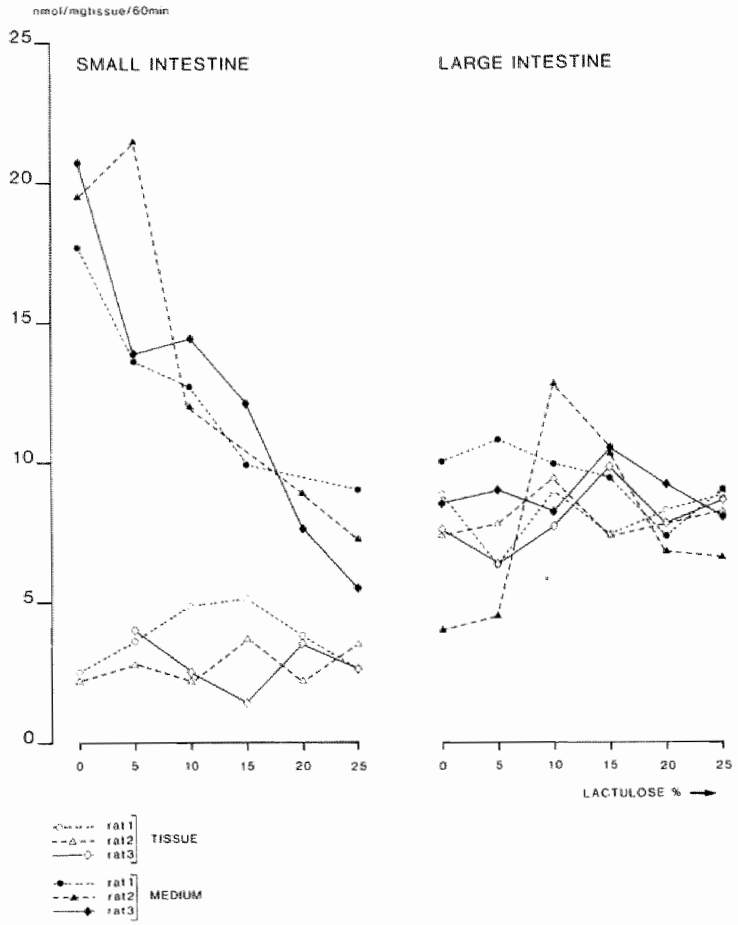

Figure 3: Ammonia production of small and large intestine of three rats after 60 min. of incubation in medium with increasing concentrations of lactulose. Ammonia was measured in the medium and in the tissue. The tissues were incubated in media with glutamine (10mM). Data are expressed in nmol/mg tissue$_{W.W.}$/60min.

This decrease was already significant ($p < 0.002$) at the lowest concentration of lactulose (5%). Ammonia production in the small intestine decreased to less than 40% of control values when 25% of lactulose was added to the medium. Ammonia concentrations in homogenates of small and large intestine did not exhibit a significant decrease when lactulose was added, nor did lactulose inhibit ammonia production in the colon.

Glutamic acid production decreased in medium of small ($p < 0.025$) and large intestine ($p < 0.02$) (fig. 4).

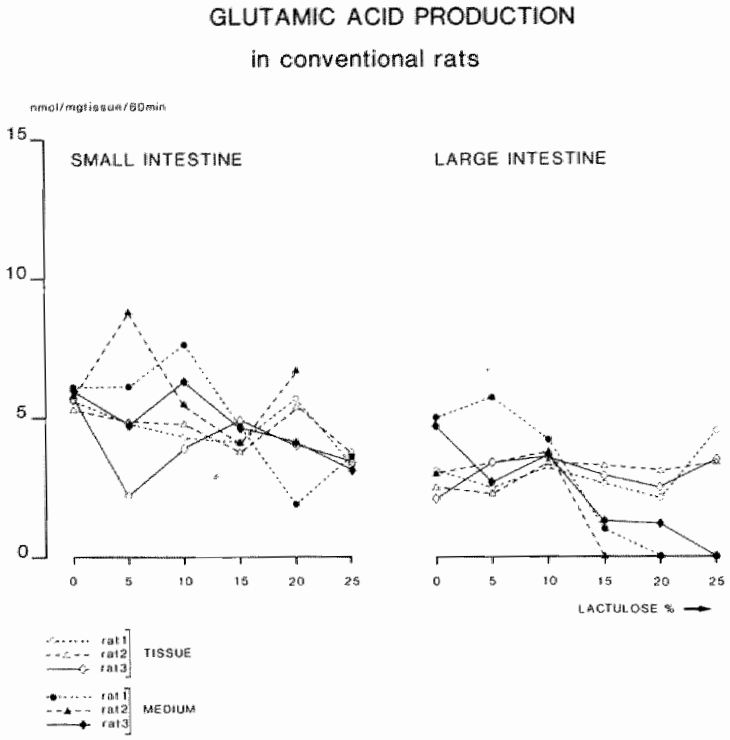

Figure 4: Glutamic acid production of the small and large intestine of three rats after 60 min. of incubation in medium with increasing concentrations of lactulose. Glutamic acid was measured in the medium and in the tissue. The tissues were incubated in medium with glutamine (10mM). Data are expressed as nmol/mg $tissue_{W.W.}$/60min.

After addition of lactulose alanine production decreased in the medium and homogenate of the ileum ($p < 0.001$ and $p < 0.001$ resp.) and not in the colon (fig. 5) (non-parametric test used).

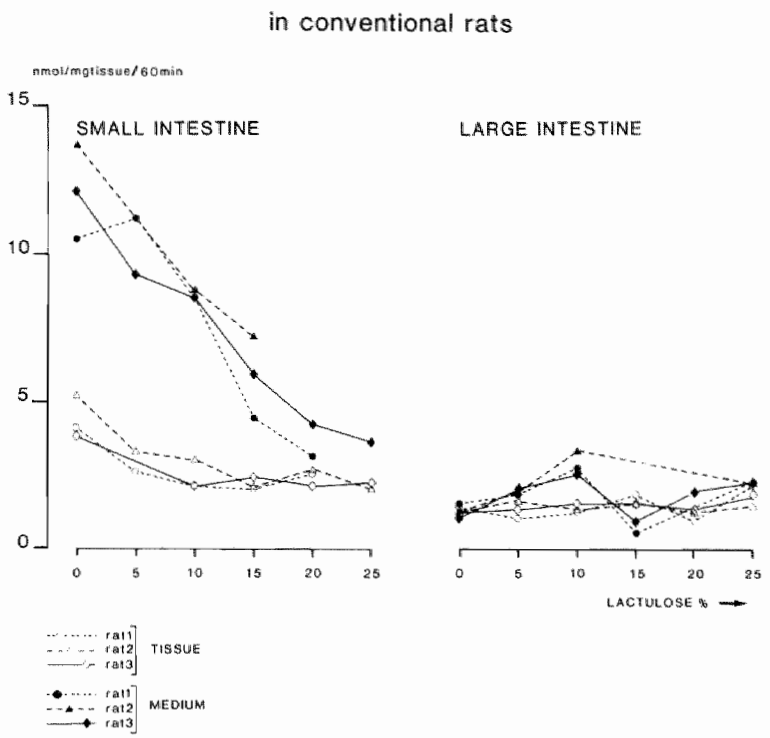

Figure 5: Alanine production of small and large intestine of three rats after 60 min. of incubation in medium with increasing concentrations of lactulose.
Alanine was measured in the medium and in the tissue. The tissues were incubated in media with glutamine (10mM). Data are expressed in nmol/mg tissue$_{W.W.}$/60min.

The effect of neomycin on ammonia production in vitro.

To study the effects of neomycin on non-bacterial ammonia production, small and large intestine were incubated with increasing concentrations of neomycin (8.1 µM - 814 µM). Neomycin achieved a reduction of the ammonia concentration in homogenate of small intestine but not in the incubation medium. No substantial effect was observed on ammonia production in the large intestine. The concentrations of alanine and glutamate in medium or homogenate of small and large intestine were not significantly affected by neomycin under these circumstances.

II.4. Discussion

The results indicate that small and large intestine exhibit a substantial capacity to metabolize glutamine in vitro. The degradation of glutamine is most likely not mediated by bacterial activity because the preparation of the tissue pieces eliminates most of the gut flora. Results therefore are in accordance with the literature where it has been demonstrated by several investigators that the gutwall can take up glutamine (9,11).

Windmueller investigated glutamine metabolism in a preparation of perfused rat intestine and found that the glutamine nitrogen taken up by the intestine could be accounted for 33% by alanine production, for 23% by ammonia production and small amounts by proline and citrulline production. Metabolism by small and large intestine was not investigated separately however. In contrast to the in vitro studies of Matsutaka (8) in which tissue pieces of 200 mg were used, our studies showed a net increase in alanine production after addition of glutamine to the incubation medium. Our results may be more representative for the in vivo situation where glutamine uptake in the gut especially yields alanine and ammonia. Hanson (21) used perfused rat jejunum and found increased alanine production when animals had fasted. We did not confirm this in our study because no significant differences in alanine production could be detected in normally fed and fasted animals. It is noteworthy that the small bowel took up more glutamine than the large bowel and released more alanine, ammonia and glutamic acid into the medium (the finding that less glutamine was taken up by colon than by small bowel is consistent with the observation that colonic tissue has a lower glutaminase activity than small bowel (22)). This is contrary to what one would expect if metabolism of these substances would be mainly bacterially mediated, because small bowel contains log 2 to 3 less bacteria.

Similarily it is striking that lactulose significantly decreases ammonia production in the small bowel and not in the colon. It thereby decreases release of alanine and glutamate. This suggests that in this in vitro study,

lactulose effectively decreases ammonia production in the small bowel through interference with intermediary metabolism. Since 2.5% lactulose increases the osmolarity of the medium with 130 mosmol one should consider the possibility that this mechanism is solely due to hyperosmolarity. Therefore, in this model we incubated sorbitol, mannitol and polyethyleen glycol with the same osmolarity. At an osmolarity of 130 mosmol no decreasing effect was noted with these substances, although lactulose inhibited ammonia for 25%. With an osmolarity of 518 m.osm all substances decreased ammonia but much less still than lactulose.

Most if not all theories regarding the effect of lactulose on ammonia production have focused on bacterial ammonia production (13,14,15,16). The results of our study do not exclude this mechanism but suggest an important role for lactulose in decreasing gut production of ammonia, alanine and glutamate by influencing intermediary metabolism in the small bowel. Neomycin decreases the concentration of ammonia in tissue homogenates of the ileum and not in the colon. No influences of neomycin on ammonia, alanine and glutamate release into the incubation medium could be noted. Interpretation of these data is difficult but, with the exception of decreased ammonia levels in small intestine homogenates, these data do not suggest a role for neomycin in decreasing metabolic ammonia generation in the gut wall. These data justify further in vivo studies in animal models to establish the role of bacterial flora and gut wall in ammonia generation and the influence of lactulose and neomycin. In addition, the role of the different parts of the gutwall in ammonia metabolism should be specified.

II.5. References

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Chapter III

METABOLIC CHARACTERISTICS OF VILLOUS AND CRYPT ENTEROCYTES OF CONVENTIONAL AND GERM-FREE RATS AND THE INFLUENCE OF NEOMYCIN AND LACTULOSE

III.1. Introduction

In recent years considerable progress has been made to identify the small intestine as an important site of glutamine utilisation, thereby contributing significantly to the respiratory fuel of the intestinal mucosa. According to Windmueller and Spaeth (1,2) ammonia, alanine, citrulline and proline are the main nitrogenous products derived from glutamine. However, these studies were carried out with an isolated perfused preparation and the site of ammonia production could not be identified. Watford and Lund (3) indicated in their studies with chicken enterocytes that of the added substrates glutamine, glucose and glutamate were the preferred fuels of respiration in the enterocyte. Presumably the enterocytes were mainly derived from the villous compartment and no information about the relative contribution of crypt epithelial cells to glutamine metabolism was obtained. Whether lactulose and neomycin influenced glutamine metabolism and ammonia production in villous and/or crypt epithelium has never been the subject of study. In chapter II we have suggested that lactulose and possibly neomycin are capable of inhibiting the glutamine dependent ammonia production. The exact site of this process was not identified. Therefore we investigated the ammonia production of villous and crypt cells and the influence of lactulose and neomycin on this ammonia production.

III.2. Material and methods

Rats.

Specific pathogen free (SPF) male and germ-free Wistar rats weighing approximately 250-300 g were obtained from the centralized experimental animal facilities of the University Biomedical Center, Maastricht. Rats were kept at 21°C and allowed free access to water and rat chow (formula SRM A120, Hope Farms, Woerden, The Netherlands). All experiments reported were carried out with cells from normally fed rats. Germ-free experiments were done under sterile conditions. Cultures of medium were performed before and after the experiments and no bacteria were present.

Isolation of villous and crypt cells.

Male Wistar rats were used for the isolation of villous and crypt cells. Small intestine was freshly removed during ether anaesthesia and the lumen was flushed with 150 ml of ice-cold saline. Further work was done at 0-4°C. Cells were prepared in principle according to the vibration technique of Harrison & Webster (4) as described by Iemhoff et al (5) and Hølsman et al (16). In short four 15 cm pieces of everted intestine were attached to the metal rods with surgical silk. The rods were exposed to longitudinal vibration following attachment to a vibromixer (type E1 Chemap, Mannedorf, Switzerland). The intestine was first vibrated (100Hz 2mm amplitude) for 1 minute in saline to remove mucus and adhering material followed by 12 minutes of vibration in isolation medium containing 0.15 M NaCl, 5 mM EDTA, 10 mM TrisHCl, pH 7.4 in order to release upper villous cells. Before harvesting the crypts, the intestine was vibrated for another 18 min to remove lower villus. This suspension was discarded. Then by the insufflation of air into the space between intestine and metal rod the pieces of intestine were dilated to expose the crypts better to the medium. By vibration for another 30 min, crypt cells could be released. Upper villous and crypt cell suspensions are centrifuged for 1 min at 300 x g (Iemhoff et al, 5). Cells were

then washed three times and centrifugated for 1 min 300 x g with a buffer containing: 128 mM NaCl, 4.7 mM KCl, 20.2 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4H_2O$, 0.33 mM $Na_2HPO_42H_2O$, 0.5 mM Naβ-hydroxybutyraat, 10.0 mM Hepes, 1.0 mM $MgCl_26H_2O$, 1.3 mM $CaCl_2$ $6H_2O$ equilibrated with O_2/CO_2 (95 : 5 v/v). Then 10 mM glucose and bovine serum albumin (BSA) 1 g/l was added. The same buffer was used for the incubation procedure. All isolation and incubation procedures were carried out with plastic material, thus minimizing cell rupture. During isolation, only a small percentage of the cells is damaged, as judged by leakage of soluble enzymes and measurements of protein and DNA synthesis (Iemhoff et al, 5; de Jonge et al, 7). Moreover both oxygen consumption rate (measured polaro graphically as reported in ref. 7) and the rate of glucose utilization in villous and crypt cells are virtually constant during 30 min of incubation under our experimental conditions (5,7 and H.R. de Jonge: unpublished observations).

Reagents.

Crystalline glutaminase L-glutamine amidohydrolase, EC 3.5.1.2. (grade V) was obtained from Sigma U.S.A.. The following were products of Boehringer Mannheim:

- Glutamate dehydrogenase (L-glutamate; NAD(P) oxidoreductase (deaminating), EC 1.4.1.3.) in 50% glycerol, from beef liver (specific activity 120 U/mg);
- L-alanine dehydrogenase from Bacillus subtilis (L-alanine: NAD oxidoreductase (deaminating), EC 1.4.1.1.). Specific activity: circ. 30 U/mg;
- β-Nicotinamide-adenine dinucleotide; NAD free acid, grade 1, 100%;
- Adenosine-5'-diphosphate (ADP) disodium salt;
- β-Nicotinamide-adenine dinucleotide phosphate, reduced (NADPH, tetrasodium salt, 98%);
- α-Ketoglutaric acid, crystallized free acid 98%;
- Lactulose (Duphalac) was obtained from Duphar (Weesp) and contained 667 mg lactulose/ml;

- Neomycin sulphate was obtained from Lundbeck, Amsterdam, The Netherlands.

Incubation procedure.

Cells were suspended in buffer (DNA content 50 µg/ml final concentration) with and without the addition of lactulose 2.5%, lactulose 10% W/V or neomycin 81.4 µM and 814 µM. Two ml cell suspension was incubated with and without glutamine 10mM at 37°C for 0 and 30 min. During the incubations the suspensions were gassed with O_2/CO_2 (95 : 5 v/v). Incubations were stopped by the addition of 200 µl perchloric acid (PCA) 60% (v/v) and directly frozen at -75° C. Metabolite analyses were carried out following neutralization of P.C.A. with KOH and removal of the insoluble $KCLO_3$ by centrifugation.

Determination of metabolites.

Ammonia was determined according to the method of DaFonseca (8). Alanine, glutamine and glutamic acid were measured in the neutralized supernatant by an enzymatic micromethod using a centrifugal analyser (Cobas-Bio, Hoffmann La Roche). For the concentrations of the solutions used, the preparations of the samples and the enzymatic assay we followed the methods of Williamson for alanine (9), of E. Bernt for glutamate (10) and of Lund for glutamine (11).
DNA content in villous and crypt suspensions were determined according to Burton (12).
Lactate dehydrogenase (LDH) was determined in the cell medium after centrifugation of the cell suspensions at 300 x g for 5 min. Total lactate dehydrogenase content of the villous and crypt cells was measured after complete lysis which could be performed by sonification for 40 sec (with intervals for 5 sec) on ice [Sonicator type Ultrasonic desintegrator 100 watt model (NV Beun - de Ronde, H.V.L.) amplitude 12 microns]. It was measured with a Boehringer test kit based on the method of Wroblewski (13).

Interassay variations.

- Alanine. The following standard concentrations were used: 16.5 - 33.0 -

82.7 and 165.0 µmol/L (n=15). The observed results were respectively 15.4 - 32.4 - 81.5 and 162.0 µmol/L. Coefficients of variation (CV) were respectively 3.5 - 3.0 - 2.5 and 2.6%.

- Glutamic acid. The following standard concentrations were used: 16.6 - 32.3 - 65.0 - 131.3 and 333.3 µmol/L (n=8). The observed results were respectively 18.3 - 35.5 - 67.9 - 130.4 and 336.0 µmol/L. CV were respectively: 9.4 - 5.1 - 4.9 - 3.4 and 0.7%.
- Ammonia curve. The following standard concentrations were used: 50.0 - 100.0 - 200.0 and 300.0 µmol/L (n=20). The observed results were respectively 51.5 - 102.0 - 200.0 and 301.0 µmol/L. CV were respectively 6.5% - 4.4% - 2.9% and 1.8%.
- DNA curve. The following standard concentrations were used: 3.0 - 6.0 - 15.0 - 22.5 - 30.0 - 45.0 - 60.0 µg/ml. The observed results were respectively 3.1 - 6.0 - 14.7 - 22.4 - 30.8 - 44.7 and 59.7 µg/ml.

Intra-assay variations.

Intra-assay variations of alanine, glutamic acid and ammonia are respectively 2.7% - 0.5% and 0.9%. The minimum detection level of alanine, glutamic acid, glutamine and ammonia in villous and crypt cells of the T30 and T0 analysis is 10 µmol/L.

The analytical mean recovery of alanine, glutamic acid and ammonia after 60 min and 30 min incubations with and without glutamine 10 mM and with and without lactulose and neomycin were for alanine, glutamic acid and ammonia respectively 100% - 99.5% and 99.8%.

III.3. Results

Ammonia and amino acid release by villous and crypt cells of conventional and germ-free rats.

Villous and crypt cells of small intestine of conventional and germ-free rats

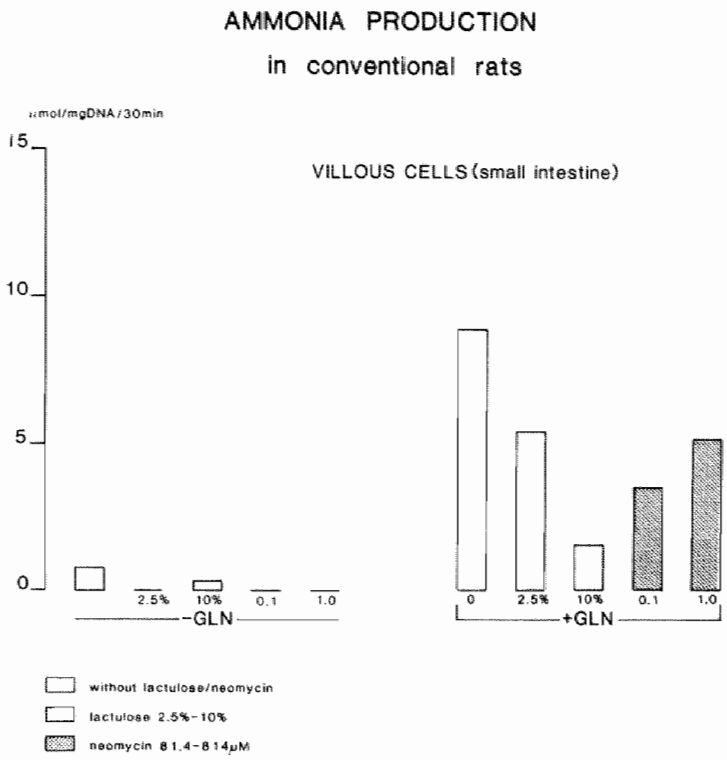

Figure 1: Ammonia production in villous cells of conventional rats incubated in Krebs Ringer buffer (± glutamine 10 mM) with and without lactulose (2.5%-10% W/V) and neomycin (81.4 µM and 814 µM) added. DNA content of each sample 50 µg/ml. Results are the mean of the duplicates and expressed in µmol/mg DNA/30min. Cells were harvested from a batch of 4 rats.

were capable of generating a measurable amount of ammonia when incubated for 30 min in vitro. This production was greatly increased when glutamine was added to the medium (fig. 1,2,3 and 4). Concomitant with this ammonia production, alanine and glutamic acid were produced by both villous and crypt cells of conventional and germ-free rats (table II and III). In conventional rats more glutamine dependent ammonia was produced by villous cells in comparison to crypt cells (expressed per µmol/mg DNA protein). In the germ-free state production was of equal magnitude. Glutamic production of villous and crypt

cells in the conventional rats was almost similar. Glutamic acid production however was roughly twice as much in the crypt cells of germ-free if compared to the villous cell production. More alanine was produced in villous cells of conventional rats than in the crypt cells. Alanine production of villous and crypt cells was the same however.

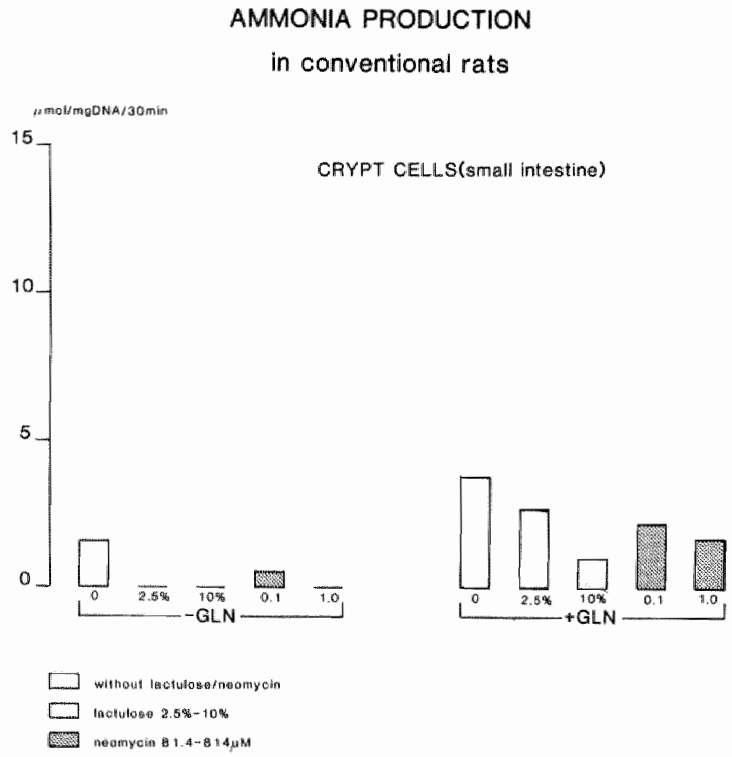

Figure 2: Ammonia production in crypt cells of conventional rats incubated in Krebs Ringer buffer (± glutamine 10 mM) with and without lactulose (2.5%-10% W/V) and neomycin (81.4 µM and 814 µM) added. DNA content of each sample 50 µg/ml. Results are the mean of the duplicates and expressed in µmol/mg DNA/30min. Cells were harvested from a batch of 4 rats.

Citrulline and ornithine were not detectable as a product of glutamine metabolism under the present experimental conditions.

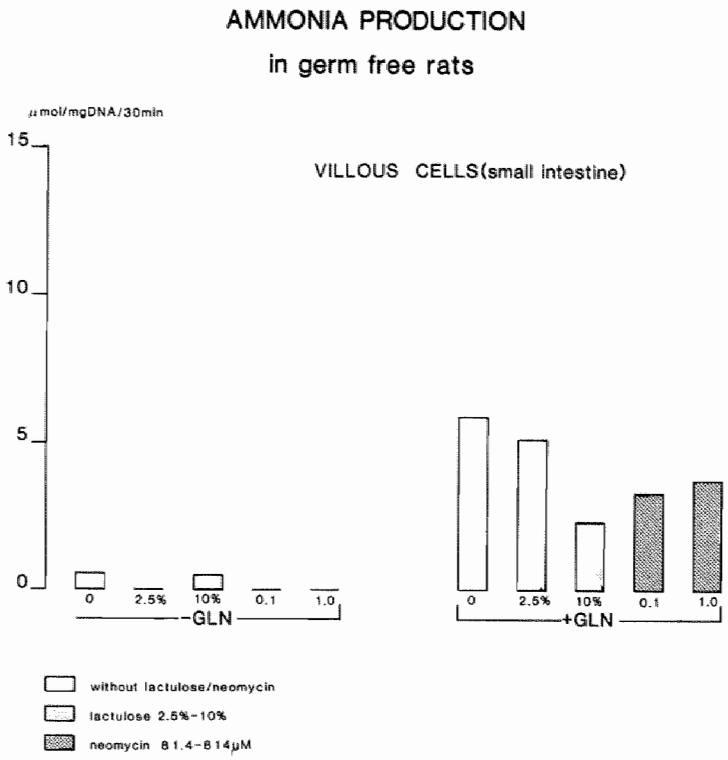

Figure 3: Ammonia production in villous cells of germ-free rats incubated in Krebs Ringer buffer (± glutamine 10 mM) with and without lactulose (2.5%-10% W/V) and neomycin (81.4 µM and 814 µM) added. DNA content of each sample 50 µg/ml. Results are the mean of the duplicates and expressed in µmolmg DNA/30min. Cells were harvested from a batch of 4 rats.

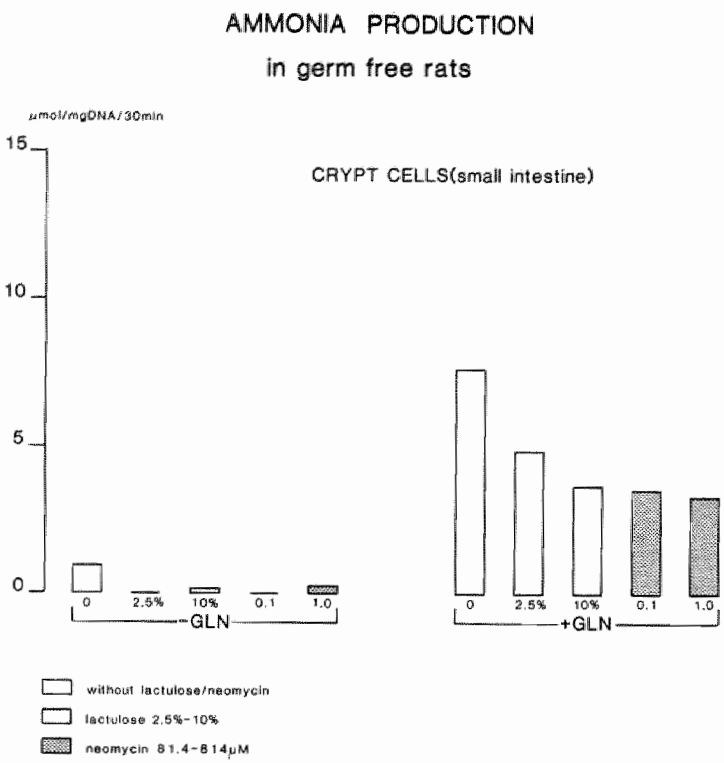

Figure 4: Ammonia production in crypt cells of germ-free rats incubated in Krebs Ringer buffer (± glutamine 10 mM) with and without lactulose (2.5%-10% W/V) and neomycin (81.4 µM and 814 µM) added. DNA content of each sample 50 µg/ml. Results are the mean of the duplicates and expressed in µmol/mg DNA/30min. Cells were harvested from a batch of 4 rats.

In order to estimate the viability of the mucosa cells after 30 min. of incubation, lactate dehydrogenase (LDH) release during incubation was determined and expressed as percentage of total cellular LDH content. It can be appreciated in Table 1 that there is a small leakage of LDH into the medium during 30 min of incubation but this release does not exceed 20% of the original content in villous and crypt cells. Lactulose (2.5%) and neomycin (81.4 µM) apparently caused only a slight increase of this LDH release. In contrast, a higher level of lactulose (10%) presumably through its effect on the osmolarity of the medium, leads to a more substantial leakage of LDH,

amounting to about 40% in the germ-free experiments.

Table I

	Buffer without Lact/Neo		Lactulose 2.5%		Lactulose 10%		Neomycin 81.4 µM		Neomycin 814 µM	
	-	+	-	+	-	+	-	+	-	+
Conventional rat										
Villous cells	6	7	12	12	20	16	16	16	10	10
Crypt cells	14	21	18	20	26	21	-	-	15	20
Germ-free rat										
Villous cells	20	20	26	25	38	39	29	27	23	24
Crypt cells	15	19		27	41	32	-	-	-	-

+ = with glutamine
- = without glutamine

Table I: Lactate dehydrogenase (LDH) measurements in the medium after 30 min of incubation expressed as percentage of the LDH content of the cells prior to incubation. Experiments were done with and without glutamine 10 mM added.

The effect of lactulose on ammonia production in vitro.

In order to study the effects of lactulose on this glutamine dependent ammonia production by the mucosa cells, lactulose was added to the medium in two different concentrations (2.5% and 10% W/V).

Lactulose decreased the ammonia production in villous and crypt cells of conventional rats already at a concentration of 2.5% (fig. 1 and fig. 2). The decrease was more dramatic at 10%. Lactulose also decreased the ammonia production in the villous and crypt cells of the germ-free rats (3,4).

The glutamic acid concentration already decreased at a concentration of 2.5% and even more so at a concentration of 10% in villous and crypt cells of conventional rats. Alanine production was decreased in conventional and germ-free rats in both the villous and crypt cells at a concentration of 10% (table II and III).

Table II

	NH_3 -	NH_3 +	Alanine -	Alanine +	Glutamic acid -	Glutamic acid +
Villous 0	0.77	8.83	0.25	5.12	0	3.39
Lac 2.5%	0	5.35	0.10	3.80	0	1.60
10%	0.32	1.50	0	1.0	0	0.20
Neo 81.4 µM	0	3.44	0.24	2.22	0	1.22
814 µM	0	5.15	0.13	2.66	0	2.49
Crypt 0	1.55	3.78	0.03	1.26	0	3.15
Lac 2.5%	0	2.67	0	1.12	0	1.68
10%	0	1.02	0	0.05	0	1.12
Neo 81.4 µM	0.52	2.18	0	0.87	0	1.39
814 µM	0	1.71	0	0.28	0	1.25

+ = with glutamine
- = without glutamine

Table II: Ammonia, alanine and glutamic acid production of conventional rats incubated in Krebs-Ringer buffer (glutamine 10 mM) for 30 min with and without lactulose (2.5% - 10% W/V) and neomycin (81.4 µM and 814 µM) added. DNA content of each sample was 50 µg/ml. Results are the mean of the duplicate experiments and expressed in µmol/mg DNA/30 min. Intestinal cells harvested from 4 rats were pooled prior to each experiment.

Table III

	NH_3 -	NH_3 +	Alanine -	Alanine +	Glutamic acid -	Glutamic acid +
Villous 0	0.54	5.89	0.43	4.09	0	1.49
Lac 2.5%	0	5.14	0.11	3.66	0	0.92
10%	0.48	2.30	0	1.60	0	0.60
Neo 81.4 µM	0	3.27	0.34	1.63	0	1.63
814 µM	0	3.76	0	2.34	0	1.48
Crypt 0	0.95	7.63	0.42	2.84	0	5.49
Lac 2.5%	0	4.85	0	2.27	0	2.57
10%	0.14	3.66	0	1.15	0	3.32
Neo 81.4 µM	0	3.53	0.32	2.45	0	0.49
814 µM	0.22	3.27	0	2.25	0	0.67

+ = with glutamine
- = without glutamine

Table III: Ammonia, alanine and glutamic acid production of germ-free rats incubated in Krebs-Ringer buffer (glutamine 10 mM) for 30 min with and without lactulose (2.5% - 10% W/V) and neomycin (81.4 µM and 814 µM) added. DNA content of each sample was 50 µg/ml. Results are the mean of the duplicate experiments and expressed in µmol/mg DNA/30 min. Intestinal cells harvested from 4 rats were pooled prior to each experiment.

The effect of neomycin on ammonia production in vitro.

In order to investigate whether neomycin was capable of inhibiting this non-bacterial ammonia production, both villous and crypt cells of conventional and germ-free rats were incubated with neomycin added in two different concentrations (81.4 µM and 814 µM). Neomycin decreased the ammonia production in villous and crypt cells of conventional and germ-free rats at both concentrations (fig. 1, 2, 3 and 4). The decrease was almost 50% in the crypt

cells of the conventional and germ-free rats at a concentration of 814 M. Glutamic acid production was decreased in both concentrations of neomycin in villous and crypt cells of conventional and in the crypt cells of the germ-free rats. Alanine production was decreased by neomycin in the villous cells of conventional and germ-free rats, in the crypt cells of conventional rats, but not in the crypt cells of the germ-free rats.

III.4. Discussion

Results indicate that the mucosal cells of the small intestine may account for a substantial part of the glutamine degradation in the gut. This degradation occurs both in villous and crypt cells and appears to be of non-bacterial origin, since these mucosal cells and the preparation isolated by the vibration procedure are virtually sterile (14). Moreover germ-free rats were likewise capable of generating ammonia in their mucosa cells. In conventional rats more ammonia was formed in the villous cells then in the crypt cells. In the germ-free state however crypt and villous cells showed an almost equal production rate of ammonia. When the production by villous and crypt cells is added together, and when the total number of crypt and villous cells isolated from conventional and germ-free rats are considered to be approximately equal (as shown by DNA content) the production capacity of ammonia is roughly equal in enterocytes of conventional and germ-free rats.

As Watford (3) and Hanson (15) concluded, the carbon skeleton of alanine can be provided by degradation of glutamine or glutamate to pyruvate and this has its significance in clearing the blood of excessive glutamine or glutamate. The uptake of glutamate by the liver is too slow to clear the high content of glutamate in the blood. Clearing of glutamate is necessary because a high concentration can be toxic to the brain (16). No citrulline and ornithine could be detected. Results are in accordance with the study by Watford (15) who also could not detect citrulline, but are in contrast to the findings of

Windmueller. Failure to detect these amino acids may be due to a very low rate of synthesis in the mucosa cells. Ross (17) reported the synthesis of ornithine or citrulline from glutamate in homogenates of mucosa from rat small intestine. The rate was 31.8 μmol/90 min/g fresh weight of tissue which is low enough to explain our failure to detect citrulline. In contrast to the literature, where neomycin and lactulose are supposed to exert their influence on hepatic coma by interfering with the bacterial flora, our results do suggest an additional effect on intermediary metabolism. Both neomycin and lactulose were capable of inhibiting the non-bacterial ammonia production together with inhibition of the production of alanine and glutamic acid.
Weber (18) determined the contribution of jejunum and ileum to portal ammonia relative to the colon in his study with dogs. He concluded that when expressed per total intestinal segment, jejunum and ileum released 28 and 22% of gut ammonia and the colon 50% into the venous blood. Ammonia released by the jejunum and ileum could be largely accounted for by the metabolism of glutamine, but in the colon for 50%. Taking our results and the results of Weber into account it is most likely that besides their possible action on the flora, lactulose and neomycin decrease ammonia by interfering with intermediary metabolism. The exact mode of action or the site where their action takes place is, at this moment, only speculative.

III.5. References

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Chapter IV

MORPHOLOGICAL EFFECTS OF HIGH DOSE NEOMYCIN SULPHATE ON THE SMALL AND LARGE INTESTINE.

IV.1. Introduction

Neomycin is an aminoglycoside antibiotic isolated from a strain of streptomyces fradiae. Oral treatment with neomycin reduces the number of aerobic gram negative rods and gram positive cocci in the intestine. It has become part of standard treatment for hepatic encephalopathy where it has beneficial effects on mental state, supposedly by the ability of neomycin to lower plasma ammonia levels. Efforts to explain the effects of neomycin by its action on bacterial intestinal flora, fail to convince because clinical state and blood ammonia levels do not correlate well with the observed alterations in faecal flora (1). Whether the effect of neomycin is only achieved by a reduction of the Enterobacteriaceae is still questionable because the aerobic flora represents only a minor fraction of the total intestinal flora.

Concomitantly it became apparent that neomycin has significant effects on intestinal function (2,3,4,5). It reduces intestinal absorption (6,7) and decreases the level of a variety of intestinal enzymes (4,8). In the present study, we wanted to evaluate changes in the morphology of the intestinal wall in the rat and the reactivity of certain mucosal enzyme systems in the course of neomycin treatment, in order to reveal a possible mechanism of neomycin in the treatment of hepatic coma on the mucosa.

Several possible ways in which the drug exerts its influence on the mucosa, include:

I. Interference with intracellular mucosal enzyme systems.

II. Interference with absorption.

III. Disturbance of the symbiotic relationship between bacterial flora and mucosa.

IV. A combination of these mechanisms.

In an endeavour to separate the effect of neomycin on gutwall mucosa from its effect on the gut, mediated by its action on the intestinal flora, germ-free rats and conventional rats were included in the study.

Several histochemical enzyme reactions were selected, each more or less representative for a specific function of the cel. To investigate whether transport function by the brush border was affected, alkaline phosphatase (AlPh) was chosen as a marker enzyme. Lysosomal distribution was observed with acid-phosphatase (AcPh). Interference with transport-function in the enterocyte was recorded by changes of the aspecific esterase staining (AE). The oxidative capacity of the cell was evaluated by changes in the NAD tetrazoliumreductase (NADHtr) and succinate dehydrogenase staining (SDH).

IV.2. Material and methods

Specific pathogen free (SPF) male Wistar rats weighing 250-300 g were used for all experiments, and were allowed free access to water and food. Germ-free animals were housed in plastic isolators. Air was filtered with bacterial filters (Miller Filters, Millipore, U.S.A.). Environmental conditions were kept constant. Entries of supplies and removal of waste products were carried out under strict aseptic conditions. Control cultures were performed weekly and on the day of termination of the experiment in the germ-free group. The diet consisted of special laboratory chow (formula SRM-A120 Hopefarms, Woerden, NL) for germ-free and conventional animals. All animals were killed by aortic exsanguination on the seventh day after daily ingestion of 500 mg neomycine or placebo. Before sacrifice, each rat was operated under ether anaesthesia. Operations were performed between 8.00 a.m. and 1.00 p.m. After an abdominal incision the proximal (14 cm from the pylorus) and distal

segments (10 cm from the ileocoecal junction) of the small intestine and segments of the colon were removed, fixed or frozen on to microtome plugs. Tissue segments were prepared for histochemical reaction on AlPh, AcPh, AE, SDH, NADHtr and for haematoxyline-eosine (HE) staining.

For AE, Est, HE, AlPh and AcPh specimens were first fixed in Holt's fixation fluid (10) for 24 hours and subsequently stored in Holt's storage medium for 48 hours. Specimens, including those for NADHtr and SDH were immersed in Freon at melting temperature. Fixed and unfixed blocks were cut with a cryostat microtome at sections of 10 µm. Per experiment we used six germ-free (3 treated + 3 controls) and six conventional (3 treated + 3 controls) animals. Three experiments were carried out.

IV.3. Results

Small intestine of conventional and germ-free rats, treated with placebo.

In the HE staining of normal rats a well developed lamina propria was seen, containing numerous lymphocytes, reticuloendothelial cells and plasma cells. Villi were well formed and regular in outline. The columnar epithelium is continuous with that of the crypt. In germ-free animals the lamina propria is poorly developed with relatively few cells in its interstices; crypts are shallow and villi are long and slender.

AlPh activity was localized at the brush border zone and juxtaapical portion of the epithelial cells in germ-free rats (fig. 1) and in conventional rats. Staining was prominent near the top and basis of the villus and negative in the crypt.

AcPh activity was high in villus epithelium and crypt cells and azodye was distributed throughout the cytoplasm of reacting cells in conventional and germ-free rats (fig. 2). A clear staining reaction was noted along the brush border of the epithelial cells of the villi. A granular distribution was observed in the apical portion of these cells.

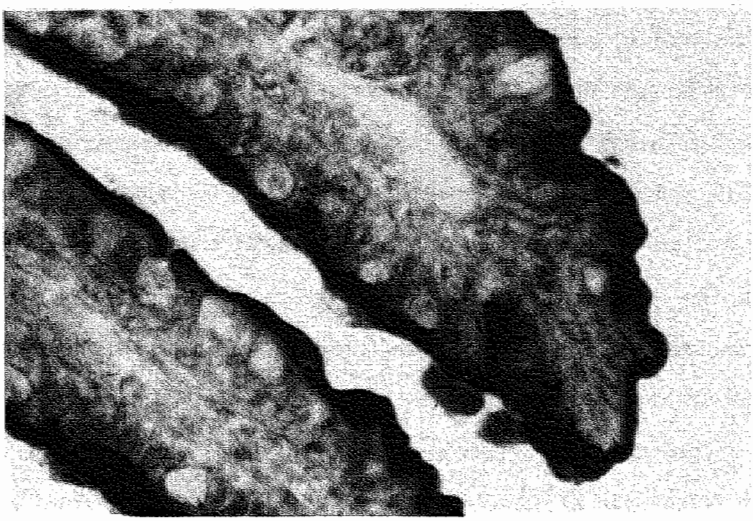

Figure 1: Alkaline phosphatase staining of the small intestine of a germ-free rat.
Continuous brush border zone.
Magnification 2340 x.

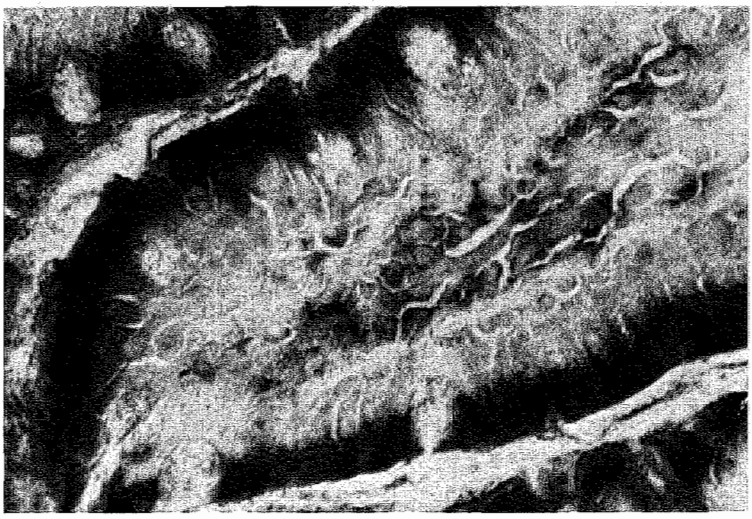

Figure 2: Acid phosphatase staining of a conventional rat. Activity in the apical region of the enterocytes.
Magnification 2340 x.

The reaction product after application of the AE reaction was distributed evenly throughout the cytoplasm of the epithelial cells. Staining reaction of the enterocytes along the basis and top of the villus was strong. Crypt surface cells reacted well in contrast to the cells in the bottom of the crypt. The epithelial cytoplasm of the basis and top of the villus reacted strongly in the NADHtr reaction. Crypt cells, both superficially and deeply situated reacted well. Granules were seen along the apical border of the enterocytes. In the SDH staining the columnar cells of both the villus top and basis, crypt surface and bottom reacted strongly in germ-free and conventional rats (fig. 3).

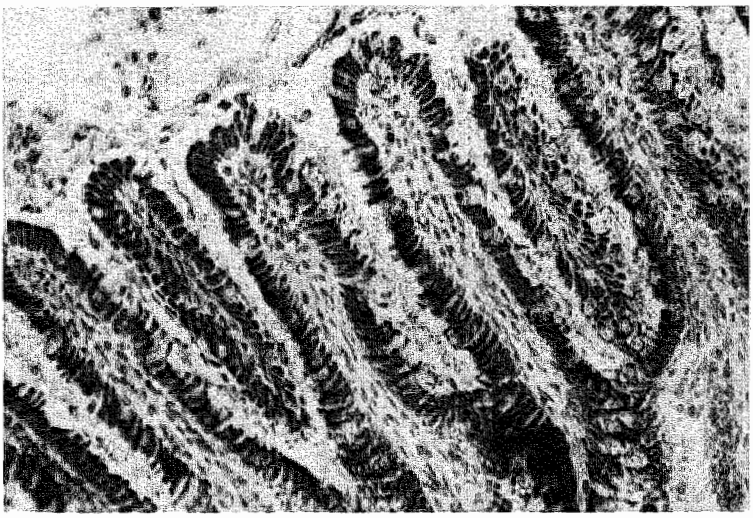

Figure 3: Succinate dehydrogenase staining of the small intestine of a germ-free rat. Even distribution of enzyme reaction product. Magnification 1560 x.

Large intestine of conventional and germ-free rats treated with placebo.

The staining reactions in the surface epithelium revealed clear columnar cells with thin striated borders and some goblet cells. The lamina propria of the large intestine as in the small intestine contains abundant diffuse lymphatic tissue. No clear AlPh reaction was noted in the crypts of the large

intestine. Epithelial cells of the crypts reacted well in the AcPh staining reaction. A strong reaction was noted in the crypt surface with the NADHtr, SDH and AE reaction. Crypt bottom reacted moderately with the NADtr and SDH reaction in contrast to a strong reaction with the AE staining.

Conventional and germ-free animals treated with neomycin.

The colon and proximal small intestine of untreated normal or germ-free animals did not show appreciable difference in staining activity with the comparable parts of animals treated with neomycin. Histologic alterations were characteristic in the rats treated with neomycin and found prominent in the distal part of the small intestine in germ-free and conventional rats (table I and II). In germ-free animals villi were widely spaced, more stubby and more irregular in outline than in untreated animals. The mucosal lining of the top of most of the villi was discontinuous. The total number of goblet cells showed an increase of approximately 50%. The top of the villi of normal rats was also irregular in outline. Alkaline phosphatase showed a change in staining pattern along the surface border of the top of the villus of both germ-free (fig. 4) and conventional rats.

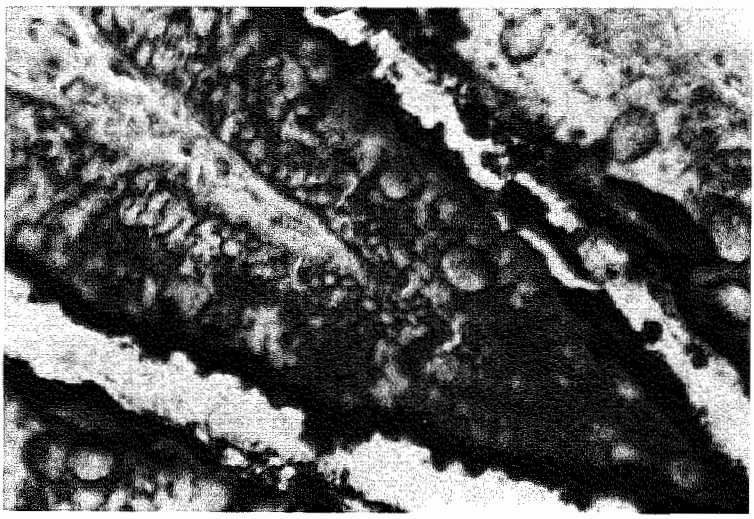

Figure 4: Alkaline phosphatase staining of the small intestine of a germ-free rat treated with neomycin. Disrupted and irregular brush border zone. Magnification 2340 x.

Table I

	Alkaline phosphatase		Acid phosphatase		N.A.D. tetrazolium reductase		Succinate dehydrogenase		Aspecific esterase	
	N	N+	N	N+	N	N+	N	N+	N	N+
Ileum villus top	++	+-	++	+-	++	+-	++	+-	++	++
Ileum villus bases	++	+-	++	+	++	+-	++	+-	++	++
Ileum crypt surface	-	-	++	+	+	+	++	+-	+	+
Ileum crypt bottom	-	-	++	+-	+	+	++	+	+-	+-
Colon crypt surface	--	--	+	+	++	++	++	++	++	++
Colon crypt bottom	-	-	+	+	+	+	+	+	++	++

N = normal rat
N+ = normal rat + neomycin

Classification
++ = strong staining
+ = well staining
+- = moderate
- = negative

Table I: The presence of several enzymes in different regions of gut epithelium.

Table II

	Alkaline phosphatase		Acid phosphatase		N.A.D. tetrazolium reductase		Succinate dehydrogenase		Aspecific esterase	
	G	G+	G	G+	G	G+	G	G+	G	G+
Ileum villus top	++	+-	++	+-	++	+-	++	+-	++	++
Ileum villus bases	+	+-	++	+	++	+-	++	-	++	++
Ileum crypt surface	+	-	++	+	+	+	++	-	+	+
Ileum crypt bottom	-	-	++	+-	+	+	++	++	+-	+-
Colon crypt surface	--	--	+	+	++	++	++	++	++	++
Colon crypt bottom	-	-	+	+	+	+	+	+	++	++

G = Germ-free rat
G+ = Germ-free rat + neomycin

Classification
++ = strong staining
+ = well staining
+- = moderate
- = negative

Table II: The presence of several enzymes in different regions of gut epithelium.

Staining activity was markedly reduced along the whole surface area. Enterocytes in the small intestine of germ-free and conventional rats after neomycin treatment reacted moderately with AcPH staining. An extensive increase in the number of goblet cells was seen along the whole border of the epithelium, which became most prominent towards the top. Activity lining the brush border was not present anymore. Granular activity was decreased and changes in distribution were seen (fig. 5). After the AE reaction, no difference in staining activity was noted in the neomycin treated animals if compared with the non-treated (table I and II).

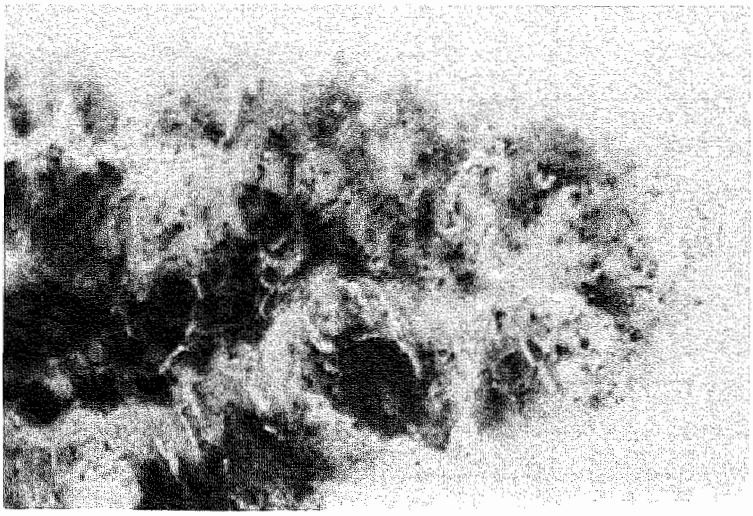

Figure 5: Acid phosphatase staining of a conventional rat treated with neomycin. Decreased activity in the apical region of enterocytes. Coarse granular distribution of activity throughout the cells. High activity in the stroma.
Magnification 2340 x.

The NADHtr reaction showed in both germ-free and conventional treated rats a decrease in staining reaction along the villi. This decrease was most prominent around the basis of the villi (table I and II). No change in staining reaction could be observed in the crypts of the animals. An extensive decrease of staining activity in the SDH reaction was noted along the villi of

the small intestine of germ-free (fig. 6) and conventional treated rats. This was most prominent along the basis of the villi.
The bottom of the crypt reacted strongly.

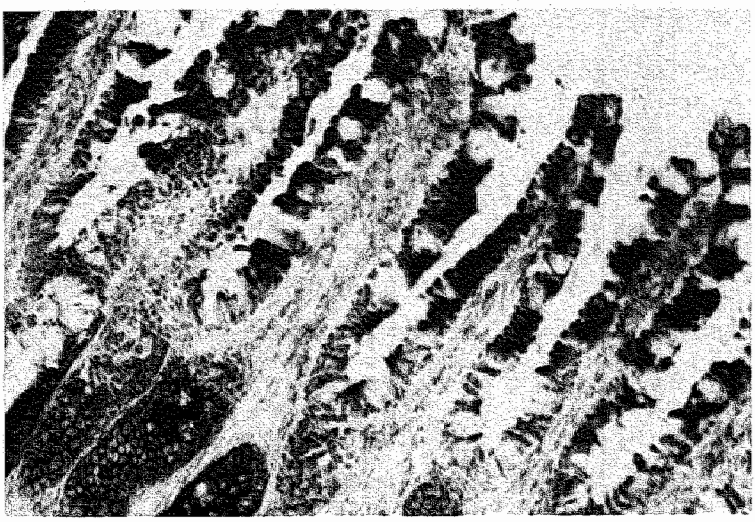

Figure 6: Succinate dehydrogenase staining of the small intestine of a germ-free rat treated with neomycin. Basal parts of the villus show decreased activity.
Magnification 1560 x.

IV.4. Discussion

In addition to a antimicrobial activity against the aerobic flora neomycin induces malabsorption and enzyme reduction. It is not clear whether there is a causal relation between these phenomena or that they are mutually independent (8,11). Examination of the small and large intestine provided a possible clue for actions of neomycin, that are not mediated via the gut flora. In agreement with earlier reports (12,13) we have shown morphological changes following orally administered neomycin recognizable with light microscopy. Neomycin decreased both in conventional and germ-free rats the activity of NADHtr, SDH, AlPh and AcPh in the small intestine. In contrast to the study of Khoury (14) with mice who demonstrated a decrease in staining reaction of

AE and NADHtr levels in the proximal small intestine and not in the distal intestine, we noted a decrease in staining activity of NADHtr in the distal part. No change in activity of AE was observed in our study. Schiraldi (15) described a decrease in reactivity of AlPh and SDH, which we could confirm in the small bowel of neomycin treated germ-free and conventional rats. This study indicates that neomycin is capable of decreasing the concentration of certain enzyme systems in conventional and germ-free rats. It suggests that neomycin has a direct effect on mucosal enzyme systems in the small intestine apart from its action on gut flora because the morphologic alterations after neomycin treatment are similar in normal and germ-free animals. Consequently no evidence in support of the third hypothesis, as referred to in the introduction, has been found. The first stage of the action of neomycin could occur at the enterocyte brush border. AlPh is attached to the membrane (17). Since AlPh reaction was decreased in this study, this finding correlates with the hypothesis that neomycin possibly exerts an action via interference with absorption. Neomycin is also likely to interfere with the intracellular metabolism of enterocytes as is shown by the results of AcPh, SDH and NADHtr reactions. As these enzymes promote reactions of a completely different nature, it appears that neomycin exerts a non-specific influence on their activity. In view of the fact (18) that neomycin disturbs protein synthesis in bacteria by binding to microsomal subunit 30s, this would support the possibility formulated in the first hypothesis, that neomycin interferes with intracellular mucosal enzyme systems, for instance by inhibition of their synthesis. It can not be concluded from our study that neomycin acts differently on villous epithelium and crypt epithelium. We have no explanation why the colon did not show a difference in staining activity after neomycin treatment. More precise ultrastructural studies are necessary to investigate the interaction of neomycin with the intestinal brush border. The effect on intracellular or membrane bound enzymes needs to be investigated by more basic biochemical study.

The results of this study indicate that explanations for the beneficial

effects of neomycin, for instance on mental state in liver disease, should not only include the antimicrobial action of neomycin but also its potential influence on absorption and possibly, on intracellular metabolism in the mucosa.

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Chapter V

THE EFFECT OF INTESTINAL FLORA MODULATION ON AMMONIA PRODUCTION IN THE RAT INTESTINE

V.1. Introduction

It has been claimed that a considerable amount of ammonia is generated in the colon and that most body ammonia originates in the gut from hydrolysis of urea by bacterial ureases (1,2) and a smaller fraction from bacterial metabolism of other nitrogenous substances in dietary residues, desquamated epithelial cells and endogenous secretions (3,4). Approximately 15-30% of the total urea pool i.e. about 7.4 g (1) is hydrolysed and the main source of urease in the colon is of bacterial origin. Urea enters the colon by passive diffusion (5). Many intestinal organisms like Bacteroides spp, bifidobacteria, Clostridia spp, Proteus spp and Klebsiella spp excrete urease, others like Escherichia coli do not split urea so that ammonia produced by these organisms must be derived from deamination of substances other than urea. Many species will produce ammonia by both mechanisms. O'Grady (7) showed that the most active of the intestinal organisms in the production of ammonia were Gram-negative aerobic rods c.q. E. coli, Klebsiella spp, Proteus spp and Pseudomonas spp. In normal healthy persons the ammonia generated in the gut is chiefly confined to the portal circulation, but in hepatic disease the liver fails to metabolize ammonia or collateral circulation exists so that ammonia enters the general circulation. This is claimed by some authors to result in chronic or recurrent neuropsychiatric disorders and ultimately in hepatic coma (8,9). Therefore a rational therapy of hepatic coma is the inhibition of ammonia production in the gut by interfering with the intestinal flora. Neomycin and lactulose are now both generally accepted in the therapy for hepatic coma and

are believed to reduce blood ammonia levels by their influence on the intestinal flora. Neomycin is mainly active against the Enterobacteriaceae (10,11) and reduces ammonia levels (12). Lactulose in a faecal incubation system influences the faecal flora by lowering the pH and stimulates incorporation of ammonia into bacterial protein (13). In vivo this would result in lowering blood ammonia levels. The question whether the effect of neomycin is only achieved by a reduction of the number of Enterobacteriaceae in the intestinal tract is still not solved because alterations of patient flora do not correlate with clinical improvement (14) and the aerobic flora represents only a minor fraction of the total intestinal flora. The largest part of ammonia has been claimed to be generated in the colon by hydrolysis of urea. This is difficult to explain because the colon is relatively impermeable to urea (15) and very little urea is entering it (16). This study was undertaken to quantitate the contribution of the aerobic and anaerobic intestinal flora in ammonia production. The quantitation of ammonia was done by selectively decontaminating conventional animals and by colonizing germ-free rats with a defined aerobic, anaerobic or mixed (aerobic + anaerobic) flora. Ammonia concentration was measured in the portal vein after occlusion of veins from the stomach and the spleen in conventional, germ-free and gnotobiotic rats. In addition, ammonia production in the effluent of small and large intestine of conventional, selectively decontaminated and germ-free animals was measured.

V.2. Material and methods

Animals.

For these studies specific pathogen free (SPF) male Wistar rats (250-300 g) were obtained from the centralized experimental animal facilities (CPV) of the University of Limburg (RL). During the experiments the animals were allowed free access to water and food. Environmental conditions were kept

constant. The diet consisted of special sterilized rat chow (formula SRM-A120 Hopefarms, Woerden, NL). Germ-free and gnotobiotic animals were housed in plastic isolators with filtered sterile air (Miller-filters, Millipore, U.S.A.). Entries of supplies and removal of waste products were carried out under strict aseptic conditions.

Gnotobiotic and selectively decontaminated rats.

From the coecum contents of SPF rats of the barrier-sustained breeding unit of the CPV of the RL, the most predominant bacterial species were isolated and after purification cultured. Gnotobiotic rats were prepared by colonizing groups of germ-free rats housed in separate isolators with several species. The gnotobiotic rats with a facultative aerobic flora (GAF) received approximately 10^8 colony forming units (CFU) by mouth of both Escherichia coli and Streptococcus faecium. The gnotobiotic rats with an obligatory anaerobic flora (GAnF) received each 10^9 CFU suspended in anaerobic transport medium of each of the following species: Clostridium perfringens, Clostridium inocuum, an unidentified Clostridium, Bacteroides ruminiculum, Bacteroides fragilis, Lactobacillus acidophilus, and an unidentified Peptostreptococcus and Bifidobacterium. The gnotobiotic rats with a mixed facultative aerobic and obligate anaerobic flora (GMF) were prepared by receiving two days after the aerobic species also the anaerobic species by the oral route.

Physiological parameters were used to assess the normalisation of the gastrointestinal tract of the three different groups of gnotobiotic rats and the influence of selective decontamination. This includes total counts of facultative aerobic and obligatory anaerobic bacteria in the coecum (table 1-A), concentrations of volatile fatty acids (VFA) (table 1-B), absence of beta-aspartylglycine in the faeces and the relative coecum weight (table 1-A).

Selective decontamination was carried out by adding antibiotics to the drinking water: 200 mg trimethoprim and 1000 mg sulfadiazine (Tribrissen^R powder, Wellcome, England), 100 mg colistine and 0.7 ml HCl per liter, in order to remove the Enterobacteriaceae.

Table I-A

	aerobes coecum lumen	anaerobes coecum lumen	R.C.W.	β-aspartyl glycine
Conventional rats	7.76 ± 0.74	9.36 ± 0.27	1	---
Germ-free rats	---	---	5	+++
GAF	9.36 ± 0.12	---	4	++
GAnF	---	10.34 ± 0.10	2	---
GMF	9.20 ± 0.11	10.90 ± 0.39	1	---
Selective decont. (-aerobe)	7.83 ± 0.60	9.55 ± 0.38	1	---
Selective decont. (-anaerob)	8.59 ± 0.49	---	2	++

Table I-A: Total number of aerobic and anaerobic bacteria (log CFU/g faeces ± SD) in coecum lumen, the relative coecum weight (RCW) and β-aspartylglycine in conventional, germ-free, selectively decontaminated rats (without enterobacteriaceae or without anaerobic flora) and gnotobiotic rats [colonized with either an aerobic (GAF), anaerobic (GAnF) or mixed flora (GMF)].

Control cultures from the faeces were done weekly until the bacteriological results were satisfactory. Totally decontaminated animals received drinking water containing trimethoprim 200 mg and sulfadiazine 1000 mg, 135 mg colistine, 250 mg amphotericine-B, 250 mg ampicilline, 1000 mg metronidazole and 50 g sucrose per liter. The rats were housed in laminar flow units and the animals received sterile rat chow. Cages were changed daily and were sterilised with the bedding before use. The animals were handled with sterile gloves.

Table I-B

	C_2	C_3	IC_4	C_4	IC_5	C_5	IC_6	C_6
Conventional rats								
CC	239 ± 17	37 ± 6	2 ± 1	122 ± 23	2 ± 0.3	14 ± 1	-	14 ± 2
IC	170 ± 75	34 ± 6	2 ± 1	106 ± 39	1 ± 0.2	11 ± 3	-	10 ± 3
Selectively decontaminated rats (- Enterobacteriaceae)								
CC	159 ± 65	28 ± 5	4 ± 0.2	111 ± 14	3 ± 1	13 ± 2	-	12 ± 1
IC	87 ± 49	12 ± 6	1 ± 1	45 ± 23	1 ± 1	4 ± 2	-	5 ± 3
Selectively decontaminated rats (- anaerobic flora)								
CC	24 ± 11	-	-	-	-	-	-	-
IC	44 ± 23	-	-	-	-	-	-	-
Gnotobiotic rats (+ anaerobic flora)								
CC	14 ± 5	4 ± 1	0.1 ± 0.1	2 ± 2	1 ± 0.2	1 ± 1	-	0.3 ± 0.2
IC	21 ± 12	1 ± 1	-	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	-	0.1 ± 0.1
Gnotobiotic rats (+ aerobic flora)								
CC	12 ± 2	-	-	-	-	-	-	-
IC	14 ± 9	-	-	-	-	-	-	-
Gnotobiotic rats (aerobic + anaerobic flora)								
CC	33 ± 23	1 ± 1	-	2 ± 1	0.3 ± 1	1 ± 1	-	1 ± 1
IC	52 ± 19	1 ± 0.2	0.1 ± 0.1	2 ± 1	-	0.4 ± 1	-	1 ± 0.1

CC = colon contents
IC = ileal contents
C_2 = acetic acid
C_3 = propionic acid
IC_4 = iso-butyric acid
C_4 = butyric acid
IC_5 = iso-valeric acid
C_5 = valeric acid
IC_6 = iso-capronic acid
C_6 = capronic acid

Table I-B: Volatile fatty acid (VFA) concentrations in mmol/L of conventional, selectively decontaminated rats (without enterobacteriaceae or without anaerobic flora) and gnotobiotic rats [colonized with either an aerobic, anaerobic, or mixed (anaerobic + aerobic) flora].

Volatile fatty acid analysis.

The volatile fatty acids (VFA) were measured in the faeces by gas liquid chromatography (GLC). The GLC-system consisted of a Packard Becker 433 gaschromatograph equipped with a dual column system and flame ionisation detectors in conjunction with a digital processor. The glass columns were conditioned overnight in the oven at 175°C with a carrier gas flow of 20 ml/min. The next day, 7 doses of 5 µl of 0.1% v/v formic acid in water were injected. During operation the injector temperature was 100°C and the detector temperature 200°C. The oven temperature was programmed to 110°C-190°C with increments of 20°C/min. This temperature programme was started 90 sec after injection of the sample and the final temperature was sustained for 5 min. Faeces was diluted 1:10 in anaerobic dilution fluid. These samples were prepared for gaschromatography by applying 1.9 ml of the sample spiked with 0.1 ml of a 200 mM stock solution of heptanoic acid, which was used as an internal standard, on to 1 ml of cationexchange resin (AG 50W-X_2, 200-400 mesh, hydrogen form, washed in water) packed on glasswool in a pasteur pipette. The sample was allowed to drain through the resin and the resin was than washed twice with 1 ml of 0.1% v/v formic acid in water. All fluid draining from the pipette was collected in a test tube and 1 µl directly injected into the gaschromatograph for analysis of VFA. The minimum detection level of each VFA (C_2-C_6) is 0.2 mmol/L and the coefficient of variation is 1.5%.

Detection of β-aspartylglycine.

By using a turrax mixer one part of faeces was thoroughly mixed with 3 parts of water. The homogenized samples were centrifugated at 2000 x g for at least 30 min. The supernatants were freeze dried at -70°C and stored at -25°C until analysis. The determination of β-aspartylglycine was performed by high-voltage paper electrophoresis. Electrophoresis was performed at pH 3.5 (pyridine/acetic acid/water, 1:10:89 by volume) for one hour at 3000 V with a high voltage electrophoresis equipment (MK41, Locarte Company, London, UK). Whatman 3 MM chromatography paper (46x57 cm) was used and samples of 5 to 50

µl were applied 1.5 cm apart at a distance of 12 cm from the margin of the paper that is to be immersed in the anode buffer compartment. Generally, 80 µl of a 25% solution of faecal supernatants was applied. Xylene Cyanol FF was added to a reference mixture of amino acids as a colourmarker. After electrophoresis the paper was dried at 70-80°C for 10-15 min and sprayed with 0.2% ninhydrin in ethanol. Subsequent heating at 70-80°C resulted in purple spots for most of the peptide-like material, except for β-aspartylglycine which yields a gray colour. Additional heating at 110°C for 10 min gives a clear blue spot for β-aspartylglycine (Welling, 1974) (17).

Bacteriological procedures.

Rats were anaesthesized with ether and immediately placed in an anaerobic glovebox. The small intestine, coecum and colon were removed and the luminal contents separated from intestine. Small segments of approximately one gram were removed from the entire thickness of the wall of the small intestine, coecum and colon and washed with prereduced anaerobic sterilized (PRAS) diluent. These samples and approximately one gram of the contents of the small intestine, coecum and colon were all separately weighed.

One part of each was thouroughly mixed with nine parts of PRAS diluent. The homogenates were serially diluted tenfold (10^1-10^5) and an aliquot of 0.036 ml of each dilution was spread over the surface of agar plates using a spiral plater (Spiral System, model B, Lameris, Utrecht, The Netherlands). The isolated intestinal flora was presumptively identified and, grouped by the use of selective and elective media. The dilutions 10^0-10^3 were spread on the following plates: 5% sheep bloodagar (oxoid Cm 55), endoagar (BBL 11199), MacConkey agar (oxoid M7) with 0.02% sodiumazide, mannitol salt agar (oxoid M85) and sheep blood agar with selective streptococcal supplement (oxoid SR74). The plates were incubated for 48 hours under atmospheric conditions at 37°C before counting the colonies. For detection of fungal overgrowth 0.1 ml of the 10^1 dilution of faeces of the decontaminated rats was spread over a Sabouraud dextrose agar (oxoid M4) with 0.4% chloramphenicol and incubated

for ten days at 37°C.

For the presumptive identification, grouping and enumeration of obligate anaerobic bacteria the dilutions 10^3-10^6 were spread over Wensinck agar (24), Wensinck agar with vancomycin (7.5 mg/L) and kanamycin (100 mg/L), bile aesculine agar (25) and egg-yolk agar with neomycin (100 mg/L) (26). These plates were incubated anaerobically at 37°C and the colonies counted after 72 hours and 7 days of incubation.

The concentrations of microorganisms were expressed as the logarithm to the base ten of colony forming units per gram (log CFU/g) of specimen. For the detection of bacteria in low numbers 0.1 ml of the 10^1 dilution was inoculated in thioglycolatice broth (oxoid M23) with additional Vit K_2 and haemine, anaerobically incubated and checked for growth during one week.

Operative procedure.

Each rat was operated under ether anaesthesia. Operations were performed between 8.00 am and 1.00 pm. After abdominal incision, rats were prepared for sampling venous effluent of the small and large intestine and for sampling effluent of the whole intestine. For sampling the effluent of the small intestine, arteries and veins of the colon were ligated and for the colon effluent, arteries and veins of the small intestine were ligated. In all models spleen, stomach and pancreas were excluded.

For sampling of the whole intestine only spleen, stomach and pancreas were excluded and blood was drawn from the vena porta.

Ammonia determination.

Ammonia was determined in plasma using the ion exchange method by Kingsly (18). The analysis of the plasma ammonia concentration with this method involves a two-stage procedure. First the ammonia is separated from other plasma constituents by using a strongly acidic cation exchange resin (Na^+K^+ form of the resin Dowex 50W - x 8, 50-100 mesh). Thereafter, quantitation of ammonia is performed by using the Berthelot phenol-hypochlorite reaction. The

following procedure is used to determine the ammonia content:

1. To 2 ml ammonia free water, 1 ml resin suspension is added together, tubes directly stoppered and placed on ice. All further handlings are performed on ice.
2. To these tubes, respectively 200 µl standard solution (eight different standard solutions from 25 to 500 µmol/L were used). 200 µl of plasma is added. The test procedure is directly started after blood collection in heparinized tubes on ice. Plasma is obtained by centrifugation for 10 min at 1000 x g (4°C) (Sorvall centrifuge).
3. The solutions are mixed during 5 min at 4°C.
4. The resin is subsequently washed with 20 ml ammonia free water. When the resin is settled the supernatant is removed. This washing procedure is repeated three times.
5. To the resin (at 20°C) 600 µl 0.1 M NaOH is added and mixed exactly for 2 min.
6. Then 1.6 ml phenol is added, mixed, directly followed by adding 1.6 ml hypochlorite and mixed again. The solution is then incubated for 15 min in a water bath (37°C).
7. After one hour the absorption at 630 nm is measured with a universal Photometer (Vitatron).

V.3. Results

Table II represents the mean portal ammonia concentrations of the effluents from small and large intestine of conventional, selectively decontaminated and germ-free animals. In conventional and germ-free rats, the small intestine releases more ammonia into portal blood than the large intestine, if a flow rate of small/large of 3:1 is taken into account (19).

Table II

	Controls Plasma	Selectively decontaminated without enterobacteriaceae Plasma	Selectively decontaminated without anaerobic flora Plasma	Germ-free Plasma
	n=5	n=6	n=6	n=6
Small intestine	426 ± 35.8	331 ± 56	169.6 ± 10.7	187 ± 13
	n=5	n=6	n=6	n=5
Large intestine	548 ± 57.4	545 ± 49	262.5 ± 24.8	186 ± 10.2

Table II: Mean portal ammonia concentration of small and large intestine in controls, selectively decontaminated (without Enterobacteriaceae or without anaerobic flora) and germ-free animals, in µmol/L (mean ± SEM).

Table III

	Conventional Plasma	Gnotobiotic aerobes Plasma	Gnotobiotic anaerobes Plasma	Gnotobiotic aerobes+anaerobes Plasma	Germ-free Plasma
Vena porta	422 ± 19.6	196 ± 20.7	266 ± 37	245.5 ± 9.5	200 ± 16.7

Table III: Mean portal ammonia concentration of conventional, gnotobiotic and germ-free rats in µmol/L (mean ± SEM) (n=5).
Ammonia concentrations were determined in the combined portal effluent of small and large intestine.

Total bacterial counts did not change significantly after decontamination of the aerobic flora in ileum, coecum and colon, although the Enterobacteriaceae were removed (table IV). The anaerobic counts expressed as log CFU/g decreased from 8.80 to 8.35 in the ileum lumen which might have been due to dilution of the luminal content during treatment (table V). No changes in total anaerobic counts were observed in the gutwall associated flora. Removal of the Enterobacteriaceae however did not result in a decreased concentration of ammonia in the portal effluent of small and large bowel (table II). Rats treated with a mixture of antibiotics during 14 days, until anaerobic bacterial cultures were negative, showed a slight decrease in aerobic counts of ileum lumen and wall, most likely due to dilution (table IV). Ammonia concentration of the venous effluent of the small intestine of this group showed a decrease from 426 to 169.9 µmol/L in the plasma (table II). Ammonia in the effluent of the large intestine decreased from 548 to 262.5 µmol/L in the plasma The animals which were colonized with an anaerobic flora (group GAF) showed an increase in portal ammonia (table III). Bacterial counts of anaerobic flora expressed as log CFU/g were equal to those of the conventional animals (table V). When germ-free animals were colonized with both aerobic and anaerobic flora (group GMF) the increase in portal ammonia was the same as in the anaerobically colonized gnotobiotic animals (group GAnF) (table III). Total bacterial counts of animals in group GMF were the same as in the conventional animals (table IV and V).

Table IV

Aerobes	Conventional	(-Enterobacteriaceae) selectively decontaminated	(-anaerobes) selectively decontaminated	Gnotobiotic + aerobes	Gnotobiotic + anaerobes	Gnotobiotic + aerobes/anaerobes
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Ileum lumen	7.94 ± 0.74	7.07 ± 0.61	5.09 ± 0.57	8.01 ± 0.28		7.19 ± 0.66
Coecum lumen	7.76 ± 0.28	7.83 ± 0.60	8.59 ± 0.49	9.36 ± 0.12		9.20 ± 0.11
Colon lumen	8.21 ± 0.25	7.41 ± 0.84	8.62 ± 0.76	9.46 ± 0.32		9.21 ± 0.15
Ileum wall	4.25 ± 0.89	4.74 ± 1.13	2.43 ± 0.28	6.26 ± 0.68		4.78 ± 0.94
Coecum wall	4.66 ± 0.77	5.48 ± 0.62	5.48 ± 0.89	6.14 ± 0.20		6.32 ± 0.12
Colon wall	4.79 ± 0.47	5.02 ± 0.42	4.57 ± 0.52	6.47 ± 0.30		6.75 ± 0.17

Table IV: Total numbers of aerobic colony forming units in small and large intestine of conventional, selective decontaminated (without enterobacteriaceae or without anaerobes) and gnotobiotic rats [colonized with an aerobic, anaerobic or mixed (anaerobic + aerobic) flora] expressed as log CFU/g (mean ± SD) (n=3).

Table V

Anaerobes	Conventional	(-enterobac.) selectively decontaminated	(-anaerobe) selectively decontaminated	Gnotobiotic + aerobes	Gnotobiotic + anaerobes	Gnotobiotic + aerobes/anaerobes
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Ileum lumen	8.80 ± 0.22	8.35 ± 0.21	-	-	7.74 ± 0.39	7.7 ± 0.54
Coecum lumen	9.36 ± 0.27	9.55 ± 0.38	-	-	10.34 ± 0.10	10.9 ± 0.39
Colon lumen	9.41 ± 0.41	9.44 ± 0.66	-	-	10.52 ± 0.27	10.5 ± 0.42
Ileum wall	5.32 ± 0.49	5.50 ± 0.74	-	-	4.95 ± 0.38	4.8 ± 1.09
Coecum wall	6.13 ± 0.24	6.48 ± 0.3	-	-	7.06 ± 0.27	7.3 ± 0.14
Colon wall	5.91 ± 0.39	5.97 ± 0.14	-	-	7.37 ± 0.11	7.6 ± 0.21

Table V: Total numbers of anaerobic colony forming units in the small and large intestine of conventional, selectively decontaminated rats (without enterobacteriaceae or without anaerobes) and gnotobiotic rats [colonised with an aerobic, anaerobic or mixed (anaerobic + aerobic) flora] expressed as log CFU/g (mean ± SD) (n=3).

V.4. Discussion

Neomycin acts mainly on aerobic bacteria, especially the Enterobacteriaceae (10,11) and is capable of lowering ammonia levels in the intestine (12). Selective decontamination, by removing the Enterobacteriaceae should result in decreased ammonia levels in the portal blood of the large intestine where the bacteria are chiefly situated, if this hypothesis is true.

The results demonstrated however that ammonia levels did not decrease substantially. The anaerobic flora however seemed to have a greater impact on ammonia generation. When the anaerobic flora was completely removed by selective decontamination, ammonia levels decreased strongly. In a second model with gnotobiotic animals, colonized with a defined aerobic and an anaerobic flora results were not completely in accordance with the selectively decontaminated rats. In group GAF ammonia levels did not increase substantially, but in group GAnF there was an increase, although the same levels as in conventional animals were not reached. Animals of group GMF with a total bacterial count equal to those of conventional animals did not reach the ammonia levels of conventional animals either. These results suggest that the given anaerobic flora plays a role in ammonia generation which is in accordance with the results of Vince (21) but it does not restore ammonia to conventional levels. Missing species might play an active role in restoring the physiology of the intestine. Although some parameters commonly used (22) to define normal physiology of the intestine reached the same levels as in conventional animals, others did not. The relative coecum weights were too high and the concentration of VFA was significantly lower than in conventional animals, but also significantly higher than in germ-free animals.

A number of species, some of which cannot yet be cultured, may be essential like for example the gutwall associated segmentated bacteria in the ileum, as described by Koopman (27). From these results it is clear that the aerobic flora plays almost no role in ammonia generation. When considering these results, the mechanism by which neomycin exerts its action is difficult to

explain. If neomycin acts on the aerobic flora and if this part of the flora does not contribute to ammonia generation, how does neomycin lower ammonia levels? Because not much is known about the effect of lactulose on the composition of the bacterial flora the results of this chapter do not provide further insight in the working mechanism of lactulose. Results can be better understood when lactulose and neomycin act also on the metabolism of the mucosa cell. If this is true lactulose and neomycin should lower ammonia levels in germ-free rats which is the case in vitro (chapter II and III). The mechanism by which lactulose and neomycin exert their influence on metabolism of the mucosal cell remains to be elucidated.

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CHAPTER VI

THE EFFECT OF NEOMYCIN AND LACTULOSE ON AMMONIA PRODUCTION IN THE SMALL AND LARGE INTESTINE IN RELATION TO THE INTESTINAL FLORA.

VI.1. Introduction

Lactulose (1-4 β-galactosido-fructose) has been used as an alternative to neomycin in patients with acute and chronic portal-systemic encephalopathy. The introduction in 1966 of lactulose promised a new therapeutic modality based on acidification of the contents of the intestinal tract. Taken by mouth, lactulose passes the small intestine unchanged and reaches the terminal ileum and colon where it is metabolized by the intestinal flora to volatile fatty acids and alcohols, the accumulation of which causes the colonic pH to fall and diarrhoea (1) to develop. It was initially suggested that acidification favoured the growth of lactobacilli (2) and other acidophilic fermentative bacteria and caused a suppression of acidophobic proteolytic bacteria (3). Quantitative studies however, have failed to confirm this change in composition of the intestinal flora (4,5). More recently it has been postulated that the beneficial response to lactulose results from the creation of a pH gradient between the extracellular fluid and the intestinal lumen which favours the trapping of ammonia (6) although studies on ammonia loss in the faeces did not show the expected increase (8). The last hypothesis that has been put forward is that lactulose might increase the metabolic activity of the intestinal flora. The intestinal flora is capable of increasing ammonia incorporation since the presence of carbohydrates as an energy source facilitates the growth of bacteria which requires the incorporation of ammonia into bacterial protein (9). As a consequence of this theory bacterial counts should increase after lactulose treatment which has not been demon-

strated. In summary, several theories have been postulated but the mechanism still remains unclear. Similarly it is difficult to understand how neomycin lowers ammonia levels. Poorly absorbed neomycin has been thought to act by reducing the activity of the microflora. It predominantly reduces the aerobic Gram-negative flora (10). Such an effect of neomycin on ammonia levels is difficult to understand since the aerobic flora represents only a minor fraction of the total flora in the intestinal tract. Moreover clinical state and blood ammonia levels do not always correlate with the observed alterations in flora induced by neomycin (11). Explanations of how lactulose and neomycin alter the flora and how this change is correlated with ammonia levels remain conflicting, because the postulated changes in flora were not confirmed in stool cultures and because at least half of the ammonia produced in the gut is derived from small bowel where much lower numbers of bacteria are present (12). Also, in studies of lactulose and neomycin treatment, gutwall associated flora was not investigated, despite studies from Savage (13) showing that the bacterial population associated with the gastrointestinal mucosa of mice differs qualitatively and quantitatively from the intestinal lumen flora. This has not been confirmed yet in man, but studies by Plant (14) revealed that the gutwall associated flora of man could not be removed by vigorous washing. To elucidate these problems, studies were undertaken to determine the influence of lactulose and neomycin on the bacterial lumen and gutwall associated flora of both the small and large intestine of rats. Animals which did not develop diarrhoea after lactulose treatment were also investigated to see whether diarrhoea is essential in the change of flora and ammonia levels. The changes in flora were correlated with the concentrations of ammonia in the portal effluent of small and large intestine separately.

VI.2. Material and methods

Animals.

Male Wistar rats (supplied by the centralized experimental animal facilities (CPV) of the University of Limburg) weighing 250-300 gr and kept under standardized conditions with free access to water and sterilized rat chow (formula SRM-A120 Hopefarms, Woerden, NL) were used for this study and divided randomly into three groups. Rats received daily neomycin (500 mg) for 10 days or 20% lactulose W/V (Duphalac, Duphar, Weesp, NL) twice daily by stomach tube. Control animals were given water as placebo. The rats receiving lactulose were divided into two groups. Rats showing diarrhoea were selected for the "lactulose with diarrhoea" group and the others in the group "lactulose without diarrhoea". All groups consisted of 6 rats.

Surgical procedures:

Each rat was operated under ether anaesthesia. Operations were performed between 8.00 a.m. and 1.00 p.m. After abdominal incision, rats were prepared for sampling of venous effluent of the small and large intestine. For sampling of the effluent of the small intestine, arteries and veins of the colon were ligated and for sampling of the effluent of the colon the arteries and veins of the small bowel. In all models, spleen, stomach and pancreas were excluded.

Ammonia analysis:

Ammonia was determined in plasma and using the indophenol blue reaction as described by Kingsly (15) (see chapter V).

Bacteriological methods.

Rats were sacrificed by the administration of an overdose of ether and immediately placed in an anaerobic glove box. Small intestine, colon and coecum were removed aseptically and luminal content separated from intestine. The contents of ileum, coecum and colon were collected separately and, after weighing, thouroughly mixed with prereduced anaerobic sterilised (PRAS)

diluent (saline with 0.05% cysteine HCl) using a turrax mixer. A segment of the entire thickness of the wall of ileum, coecum and colon was cut and washed three times in PRAS diluent to remove the intestinal contents and after weighing, also mixed with PRAS diluent to assess the total colony forming units of gutwall associated bacteria per gram tissue, and homogenized with a turraxmixer.

All homogenates were serially diluted tenfold (10^1-10^6) and an aliquot of 0.036 ml of each dilution was spread over the surface of plates using a spiral plater (Spiral System Model B, Lameris, Utrecht, The Netherlands). The facultative aerobic microflora was quantitated, presumptively identified and grouped by plating the dilutions 10^1-10^3 onto selective and elective plates. Five percent sheepblood agar (Oxoid CM 55) was used for the total count of facultative aerobes; endoagar (Oxoid CM 479) for counting coliforms; sheepblood agar with antibiotic supplement for selection of streptococci (Oxoid SR 74); MacConkey agar (Oxoid CM 7) with 0.2 g/L sodiumazide for enterococci and mannitol salt agar (Oxoid CM 85) for staphylococci. Also plates were inoculated with 0.1 ml of the homogenates spread over the surface for detection of Pseudomonas spp: pseudomonas selective medium (CM 457), and yeasts: Sabouraud dextrose agar (Oxoid CM 41) with 0.4% chloramphenicol. All plates were incubated for 48 hours at 37°C under atmospheric conditions before counting the colonies. The Sabouraud plates were also read after one and two weeks. For the total obligatory anaerobic counts dilution 10^3-10^6 were inoculated on Wensinck medium (16) using the spiral plater. Anaerobic Gram-negative rods were counted on a Wensinck medium containing kanamycin (100 mg/L) and vancomycin (7.5 mg/L). A selective medium containing bile salts and aesculine was used for quantitation of the Bacteroides fragilis group (17) and egg yolk agar (18) with neomycin (10 mg/L) for Clostridium species. All plates for anaerobes were incubated in an anaerobic glove box at 37°C in an atmosphere containing 10% H_2, 10% CO_2 and 80% N_2. The plates were counted after 48 hours and after one week.

VI.3. Results

We were able to culture a significant number of aerobic and anaerobic bacteria from the small intestine (table I and II).

The number was relatively small however, when compared to the numbers of bacteria cultured from coecum and colon. Lower numbers of bacteria were present in the proximal part than in the distal part of the small intestine. This was true for both gutcontent and gutwall.

Table I

	Controls	Lactulose + diarrhoea	Lactulose - diarrhoea	Neomycin
Aerobes	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Ileum lumen	7.94 ± 0.74	6.69 ± 0.87	7.67 ± 0.78	5.45 ± 0.35
Coecum lumen	7.76 ± 0.28	8.41 ± 0.33	8.32 ± 0.58	6.96 ± 0.67*
Colon lumen	8.21 ± 0.25	8.68 ± 0.41	8.50 ± 0.50	7.21 ± 0.66*
Ileum wall	4.25 ± 0.89	4.49 ± 0.41	4.26 ± 0.82	5.32 ± 1.66
Coecum wall	4.66 ± 0.77	5.85 ± 0.69	5.55 ± 0.76	5.62 ± 1.65
Colon wall	4.79 ± 0.42	5.90 ± 0.66	5.46 ± 0.91	4.44 ± 0.78
Anaerobes	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Ileum lumen	8.80 ± 0.22	8.37 ± 0.13*	8.56 ± 0.52	7.67 ± 0.24
Coecum lumen	9.36 ± 0.27	9.99 ± 0.61	10.05 ± 0.54	10.10 ± 0.05*
Colon lumen	9.41 ± 0.41	9.91 ± 0.40	10.33 ± 0.61	9.97 ± 0.34
Ileum wall	5.32 ± 0.49	6.28 ± 0.28	5.68 ± 0.08	7.05 ± 0.48*
Coecum wall	6.13 ± 0.24	7.27 ± 0.61	7.62 ± 0.36	7.51 ± 0.34*
Colon wall	5.91 ± 0.39	6.26 ± 0.31	7.45 ± 0.19	7.67 ± 0.16*

* $p < 0.05$

Table I: Total number of bacteria (aerobes and anaerobes) cultured from the lumen and wall of the small and large intestine in controls, lactulose and neomycin treated rats expressed as log CFU/g (mean ± SD) (n=3).

Table II

	Lumen Proximal	Lumen Mid	Lumen Distal
Aerobes	7.78 ± 0.44	8.19 ± 0.25	8.41 ± 0.3
Anaerobes	8.28 ± 0.38	8.49 ± 0.23	9.07 ± 0.04
	Wall Proximal	**Wall Mid**	**Wall Distal**
Aerobes	4.30 ± 0.15	5.08 ± 0.49	5.26 ± 0.16
Anaerobes	4.64 ± 0.19	5.30 ± 0.52	5.31 ± 0.23

Table II: Total number of bacteria (aerobes and anaerobes) cultured from the lumen of the small intestine in the proximal, mid and distal part, expressed as log CFU/g (mean ± SD) (n=3).

The influence of lactulose on the intestinal flora.

Total counts of flora of animals with lactulose are represented in table I. Lactulose did not change the total aerobic count in the small and large intestine. It did however lower the total numbers of obligatory anaerobic bacteria in the lumen of the ileum. With regard to the specific cultures of aerobes and anaerobes it is noteworthy that lactulose increased the total number of streptococci (table III) in the lactulose with diarrhoea and without diarrhoea group. In the lactulose without diarrhoea group a slight increase in the number of gutwall associated Gram-negative anaerobic rods was detected (table IV) and in the number of clostridia inside the lumen and wall of the coecum and the colon (table V).

Table III

	Conventional rat	Lactulose + diarrhoea	Lactulose - diarrhoea	Neomycin
Ileum lumen	7.39 ± 0.98	7.17 ± 0.34	7.55 ± 0.51	- *
Coecum lumen	6.58 ± 0.45	8.20 ± 0.27*	7.70 ± 0.36*	- *
Colon lumen	7.06 ± 0.3	7.86 ± 0.23*	8.01 ± 0.49*	- *
Ileum wall	3.85 ± 1.24	4.39 ± 0.32*	3.89 ± 0.65	- *
Coecum wall	3.68 ± 0.69	5.19 ± 0.68*	5.17 ± 0.35*	- *
Colon wall	3.35 ± 0.63	4.17 ± 1.22*	5.17 ± 0.68*	- *

* $p < 0.05$

Table III: Total number of streptococci, cultured from the lumen and wall of the small and large intestine in controls, lactulose and neomycin treated rats expressed as log CFU/g (mean ± SD) (n=3).

Table IV

	Controls	Lactulose + diarrhoea	Lactulose - diarrhoea	Neomycin
Ileum lumen	7.04 ± 0.17	6.84 ± 0.3	6.86 ± 0.86	6.82 ± 0.11
Coecum lumen	8.60 ± 0.31	9.19 ± 0.66	8.96 ± 0.84	9.73 ± 0.21*
Colon lumen	8.80 ± 0.17	8.74 ± 1.07	9.15 ± 0.71	9.59 ± 0.36*
Ileum wall	<4.38 ± 0.06	<4.71 ± 0.4	5.03 ± 0.31	5.10 ± 0.67*
Coecum wall	5.29 ± 0.17	5.97 ± 0.94	6.77 ± 0.82	6.49 ± 0.26*
Colon wall	5.44 ± 0.35	5.93 ± 0.79	6.65 ± 0.57	7.17 ± 0.12*

* $p < 0.05$

Table IV: Total number of Gram-negative anaerobic rods cultured from the lumen and wall of small and large intestine in controls, lactulose and neomycin treated rats, expressed as log CFU/g (mean ± SD) (n=3).

Table V

	Conventional	Lactulose - diarrhoea	Neomycin
Ileum lumen	7.67 ± 0.17	7.34 ± 0.82	4.52 ± 0.67*
Coecum lumen	4.56 ± 1.65	7.78 ± 0.08*	5.53 ± 0.22
Colon lumen	5.99 ± 1.26	9.68 ± 0.04*	5.32 ± 0.17
Ileum wall	4.36 ± 0.45	4.25 ± 0.57	2.15 ± 1.90
Coecum wall	4.56 ± 0.59	5.44 ± 0.93*	3.59 ± 0.37*
Colon wall	<3.70 ± 0.47	5.43 ± 0.98*	4.12 ± 0.26

* $p < 0.05$

Table V: Total number of clostridia cultured from the lumen and wall of small and large intestine in controls, lactulose without diarrhoea and neomycin treated rats expressed as log CFU/g (mean ± SD) (n=3).

The influence of neomycin on the gut flora.

Neomycin decreased the total aerobic numbers in the lumen of the coecum and colon. Numbers in the ileum lumen also decreased although this was not significant (table I). From the qualitative cultures, it appeared that neomycin considerably lowered the number of Enterobacteriaceae (table VI), enterococci (table VII) and staphylococci. In the anaerobic cultures an increase in the total number of anaerobic bacteria was detected in the lumen of the coecum (table 1), the wall of the ileum, coecum and colon. This increase was due to an increase of the Gram-negative anaerobic rods (table IV), mainly the Bacteroides fragilis group (table VIII) although no significance was reached for the total bacterial numbers in the colon lumen and ileum lumen (table I).

Table VI

	Controls	Neomycin treated rats
Ileum lumen	5.23 ± 1.26	< 1*
Coecum lumen	6.06 ± 0.51	< 1*
Colon lumen	6.30 ± 0.31	< 1*
Ileum wall	<2.45 ± 0.85	< 1*
Coecum wall	3.44 ± 1.10	< 1*
Colon wall	3.05 ± 0.46	< 1*

* $p < 0.05$

Table VI: Total number of Enterobacteriaceae cultured from the lumen and wall of small and large intestine in controls and neomycin treated rats, expressed as log CFU/g (mean ± SD) (n=3).

Table VII

	Control	Neomycin treated rats
Ileum lumen	6.42 ± 0.23	< 1*
Coecum lumen	6.31 ± 0.04	< 1*
Colon lumen	6.70 ± 0.28	< 1*
Ileum wall	<2.62 ± 0.70	< 1*
Coecum wall	3.30 ± 0.52	< 1*
Colon wall	2.97 ± 0.86	< 1*

* $p < 0.05$

Table VII: Total number of enterococci cultured from the lumen and wall of small and large intestine in controls and neomycin treated rats, expressed as log CFU/g (mean ± SD) (n=3).

The influence of neomycin and lactulose on portal ammonia concentrations.

Table IX represents the mean portal ammonia concentrations of small and large portal effluent in conventional, lactulose treated and neomycin treated rats. Lactulose lowered the portal ammonia concentration in the effluent of the small and large intestine in plasma if compared to controls. The decrease was most significant in the small bowel effluent where it reached a total decrease of 36%. Ammonia concentration in the portal effluent of the colon showed a decrease of only 25%. In the lactulose group without diarrhoea, plasma from the portal effluent of the small intestine decreased, but in the large bowel a significant rise in plasma levels occurred despite the low pH. Neomycin decreased plasma levels in the small and large bowel portal effluent.

Table VIII

	Control	Neomycin treated rats
Ileum lumen	5.28 ± 0.40	6.81 ± 0.17*
Coecum lumen	7.99 ± 0.73	9.25 ± 0.27*
Colon lumen	8.08 ± 0.93	9.18 ± 0.59*
Ileum wall	<4.38 ± 0.06	5.03 ± 0.73*
Coecum wall	4.47 ± 0.65	6.46 ± 0.25*
Colon wall	<4.67 ± 0.49	6.32 ± 0.46*

* $p < 0.05$

Table VIII: Total number of Bacteroides fragilis cultured from the lumen and wall of small and large intestine in controls and neomycin treated rats expressed as log CFU/g (mean ± SD) (n=3).

Table IX

	Controls Plasma	Lactulose + diarrhoea Plasma	Lactulose - diarrhoea Plasma	Neomycin Plasma
Small bowel	426 ± 35.8 pH = 6.8	270 ± 12.8 pH = 5.3	334 ± 45.7 pH = 6.1	135.3 ± 16.5 pH = 6.7
Large bowel	548 ± 57.4 pH = 6.8	408 ± 70.7 pH = 4.9	758 ± 56.7 pH = 5.6	237.2 ± 58.5 pH = 6.7

Table IX: Mean portal ammonia concentration of small and large intestine in conventional, lactulose and neomycin treated rats in µmol/L (mean SEM) (n=5).

VI.4. Discussion

The changes in bacterial numbers in small and large intestine caused by lactulose and neomycin can not explain the significant decrease in ammonia levels in the portal vein. Lactulose significantly lowered the plasma ammonia in the small and large portal effluent, when diarrhoea was present. Lactulose however did not drastically change the flora of the small and large intestine in the lumen nor the flora associated with the gutwall. Our study does not support a role for the low pH, due to breakdown of lactulose, in the ammonia lowering effect of lactulose, which is in accordance with the study of Vince (24). In the lactulose with diarrhoea group we measured a pH of 5.3 and 4.9 together with a decrease in ammonia both in small and large intestine. The decrease in pH in this group could explain the ammonia decrease in small and large bowel portal effluent although in the lactulose without diarrhoea group a low pH of 5.6 did not result in a decrease in ammonia content in the effluent of the large intestine. In contrast, ammonia rose from 548 to 758 µmol/L in plasma. It emerges from these data that diarrhoea has a greater impact on decreasing ammonia levels than the pH has. If the hypothesis of

Vince (9), that lactulose serves as an energy source, facilitating the growth of bacteria and the incorporation of nitrogen into bacterial protein, is true, it should result in an increase in the total number of bacteria. In this study no increase in the total number of bacteria after lactulose treatment was noted, but this may not have been noted because of the effects of dilution. However, in the lactulose treated group without diarrhoea an increase in the total number of anaerobic bacteria occurred in the lumen and wall of the coecum and colon (table I). According to the hypothesis of Vince this should have resulted in a decrease in ammonia concentration in plasma of the portal effluent of the large intestine. An increase occurred however (table IX) which casts doubt on the validity of the hypothesis of Vince. The possibility that in the lactulose with diarrhoea group an increased bacterial growth rate exists but that this is not noted due to dilution, can only apply to the luminal flora, since the gutwall associated flora does not change after dilution, not even after vigorous washing (14). Yet in this study no increase in bacterial number of the gutwall associated flora was detected in the lactulose with diarrhoea group. Neomycin decreased the total aerobic count in the coecum lumen and in the lumen of the colon (table I). In the anaerobic cultures it only decreased bacterial counts in the ileum lumen which might be explained by dilution. Neomycin acts on the Enterobacteriaceae and enterococci which is in accordance with the results of Imler (10). Taking these results into account, it does not adequately explain the ammonia lowering effect of neomycin, because the aerobic flora represents only a small fraction of the total number of bacteria and moreover, the anaerobic flora is more active in ammonia production than the aerobic flora (24). Furthermore, we demonstrated in the previous chapter that the aerobic flora does not contribute significantly to ammonia production. Also in cirrhotic patients, where urea splitting bacteria e.g. Klebsiella and Proteus spp increase in number (23), the ammonia lowering effect due to the effect of neomycin on these species, can not explain the clinical improvement because the colon is relatively impermeable to urea (20) and only small amounts of

urea are available in the colon lumen (21). Wrong even demonstrated that exogenous urea is not, as has generally been assumed, the major source of ammonia (22). Consequently the mechanism of action of lactulose and neomycin can not satisfactorily be explained by changes in the gut flora, especially not since both neomycin and lactulose decrease ammonia levels in the portal effluent of the small intestine, although relatively few bacteria are present. When the ammonia lowering effect of these drugs is not due to their effect on the intestinal flora, one might consider the fact that they may have an impact on nonbacterial metabolism. Weber (12) already indicated that in dogs 75% of the ammonia production in the gastrointestinal tract is glutamine dependent. Therefore, the possibility should be considered that the therapeutic effect of these drugs is also based on effects of nonbacterial metabolism and that studies with lactulose and neomycin on nonbacterial metabolism production are needed to test this hypothesis.

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Chapter VII

PORTAL ARTERIAL DIFFERENCES FOR AMINO ACIDS AND AMMONIA ACROSS THE INTESTINE IN CONVENTIONAL AND GERM-FREE RATS AND THE INFLUENCE OF LACTULOSE AND NEOMYCIN.

VII.1. Introduction

Ammonia generation in the gut is still believed to result from bacterial degradation of luminal nitrogen derived from diet, desquamated epithelial cells and hydrolysis of urea (1,2,3,4).

Bacterial ammonia generation is so firmly established that virtually all treatment for the hepatic coma syndrome has been directed towards altering the role of bacteria within the colon. However, there is ample evidence to suggest that there are sources of gut ammonia production not attributable to bacterial metabolism within the intestinal tract, but to intracellular non bacterial metabolism in the mucosa (5,6). Windmueller indicated that this ammonia production is glutamine dependent. Uptake of glutamine by rat small intestine, derived either from the lumen or from the blood resulted in the appearance of ammonia, alanine, ornithine, citrulline, glutamate, and proline in the venous effluent of the gut (7). Whether this metabolic process also takes place in the colon has not been investigated extensively, although Weber in his study indicated that in dogs 50 percent of ammonia released by the colon can be accounted for by the uptake of glutamine (8). Neomycin and lactulose are both used in hepatic coma and are generally accepted to have beneficial effects (9,10). Neomycin is known to exert its action mainly on the aerobic part of the flora, which represents only a small part of the total flora (11). Lactulose decreases pH resulting in decreased production of ammonia by gut flora and decreased absorption of ammonia by gut mucosa. It

also acts as a laxative and according to Vince serves as an energy substrate for bacteria thereby facilitating the assimilation of NH_3 into bacterial protein (12). These explanations do not take into account the suggestions in the literature indicating that a large part, possibly half to three quarters of the total ammonia production in the intestinal tract, is not bacterially mediated. Moreover according to Weber 50% of the total ammonia production originates in the small intestine where relatively few bacteria are present (8). The aim of the study was:

1. to measure the ammonia production in small and large bowel respectively in conventional and germ-free rats;
2. to determine the influence of lactulose and neomycin on ammonia production.

VII.2. Material and methods

Animals.

Male Wistar (specific pathogen free) SPF rats weighing 250-300 g were used and kept at 21°C and allowed free access to water and rat chow. Germ-free rats were housed in plastic isolators. Air was filtered with bacteria-filters (Miller-filters, Firma Millipore, U.S.A.). Enviromental conditions were kept constant. Entries of supplies and removal of waste products were carried out under strict aseptic condition. The diet consisted of special laboratory chow (formula SRM-A120, Hope Farm, Woerden) prepared for germ-free and SPF animals. Rats were given lactulose (2x2.5 ml/day of a solution of 20% W/V) (Duphalac, Duphar, Weesp, NL) or neomycin 2x2.5 ml (10 g/100 ml) (Lundbeck, Amsterdam, NL) by stomach tube for seven days. Controls were given water as placebo in the same manner.

Surgical procedure.

Each rat was operated under ether anaesthesia. Operations were performed between 8.00 a.m. and 1.00 p.m. After laparotomy porta-arterial differences

from the venous effluent of the small and large intestine. For sampling the effluent of the small intestine, arteries and veins of the colon were ligated and for sampling the effluent of the colon, the arteries and veins of the small bowel. In the germ-free animals no distinction was made between portal blood of colon and small intestine, but the portal effluent of both small and large bowel was sampled. In all animals stomach, spleen and pancreas were excluded. Ammonia, citrulline, ornithine, glutamate, glutamine and alanine were measured in plasma. Amino acids were determined on an automatic analyzer (LKB 4400). The coefficient of variation (CV) is smaller than 1%.

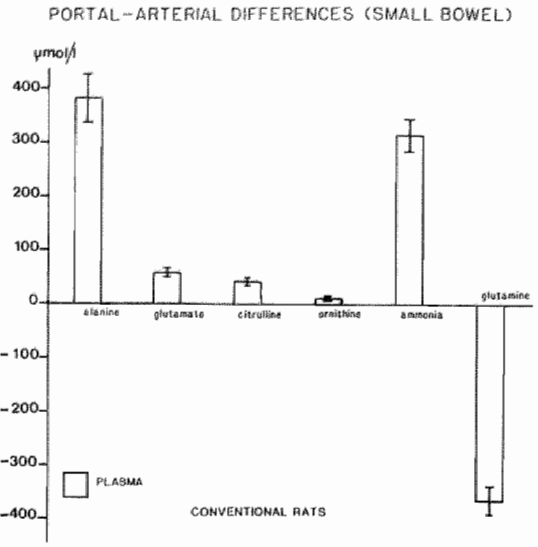
Ammonia determination.

Ammonia was determined in plasma using the ion exchange method by Kingsley (14) (see chapter V).

VII.3. Results

Results indicate when positive P-A differences are expressed as production and negative P-A differences as uptake, that small and large intestine of conventional and germ-free rats generate ammonia, alanine, citrulline, ornithine and glutamate and take up glutamine (fig. 1 and 2).

The small intestine of germ-free animals generates roughly an equal amount of alanine and ornithine, but less ammonia and glutamate and takes up less glutamine if compared to the conventional rats. The colon of the germ-free animals generates more alanine than the conventional colon. However, equal amounts of glutamate, citrulline and ornithine were produced. Much less ammonia was produced by the colon of the germ-free animal if compared to the conventional rats and less glutamine was taken up by the colon in the germ-free animals.



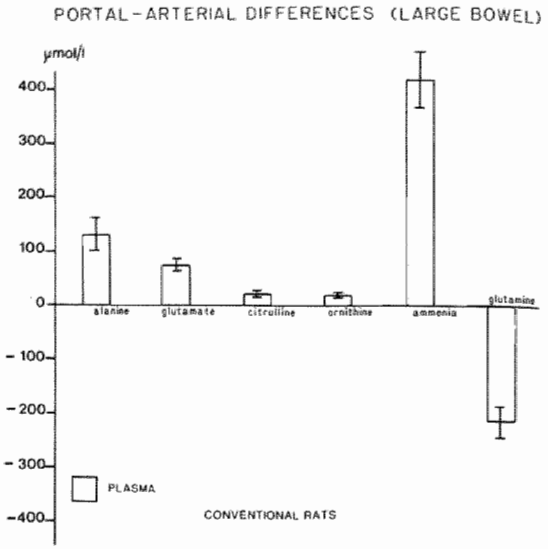
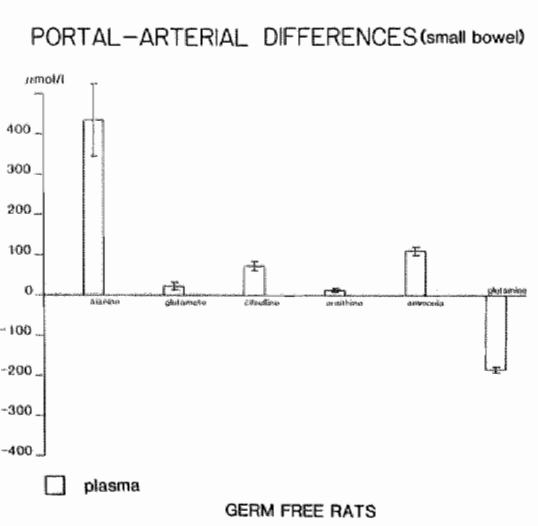


Figure 1: Portal-arterial differences of amino-acids and ammonia in plasma of conventional male Wistar rats (fed state). Portal concentrations were selectively measured in the portal effluent of the small and large intestine. Positive and negative values represent output and uptake respectively in µmol/L (mean ± SEM).



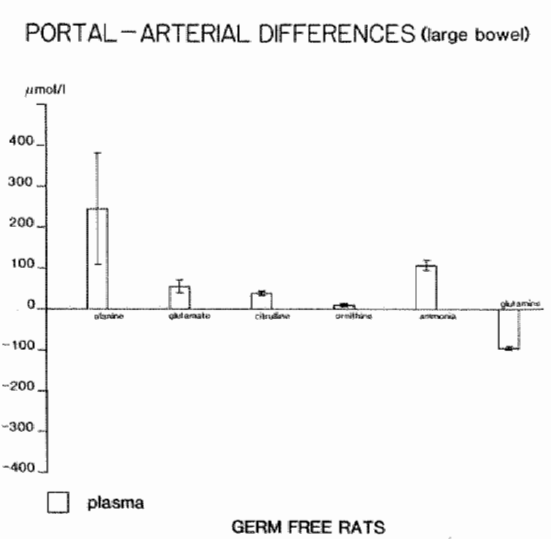


Figure 2: Portal-arterial differences of amino-acids and ammonia in the plasma of germ-free male Wistar rats (fed state). Portal concentrations were selectively measured in the portal effluent of the small and large intestine. Positive and negative values represent output and uptake respectively in µmol/L (mean ± SEM).

Small intestine of conventional and germ-free rats generate more ammonia, alanine, glutamate, ornithine, citrulline, and take up more glutamine than the large intestine, when a flow rate of small : large of 3 : 1 is taken into account (13).

The influence of lactulose and neomycin on the portal-arterial differences of conventional animals.

Fig. 3 represents the portal-arterial ammonia concentration in plasma of the control group, the lactulose group with and without diarrhoea and the neomycin treated group.

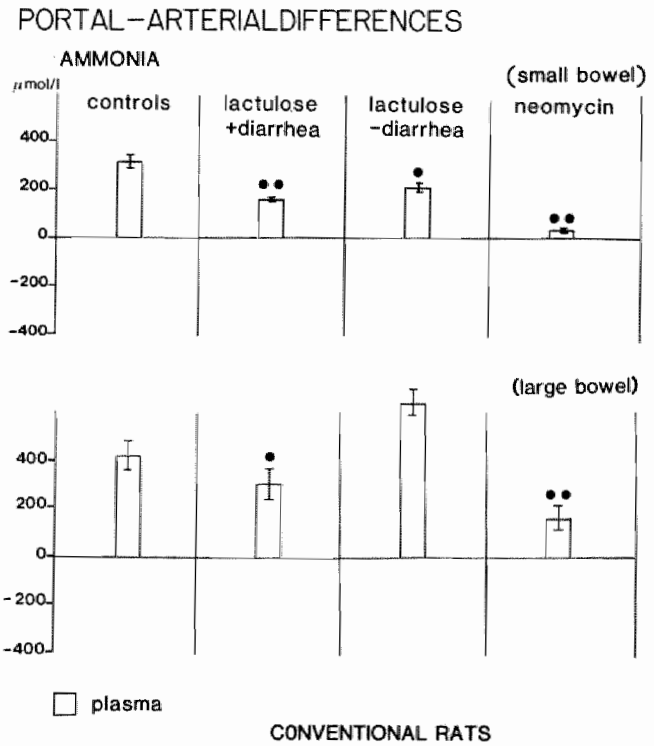


Figure 3: Portal-arterial ammonia concentrations in the small and large intestine of conventional rats (fed state) measured in plasma of control, lactulose and neomycin treated rats. Positive and negative values represent output and uptake respectively in µmol/L (mean ± SEM).

* $p < 0.05$; ** $p < 0.01$.

As indicated by a positive P.A. difference, a significant amount of ammonia is generated in the small intestine. This ammonia production of the small intestine is decreased after treatment with lactulose and neomycin.

In the colon a significant decrease in ammonia production was noted in the plasma of the lactulose with diarrhoea group and in the plasma of the neomycin group. An increase in plasma ammonia concentration was noted in the lactulose without diarrhoea group, suggesting that the ammonia not taken up by the small bowel is now taken up by the colon.

Production of glutamate in the small intestine (Fig. 4) was decreased in the plasma of the lactulose group with and without diarrhoea and in the plasma of the neomycin treated group.

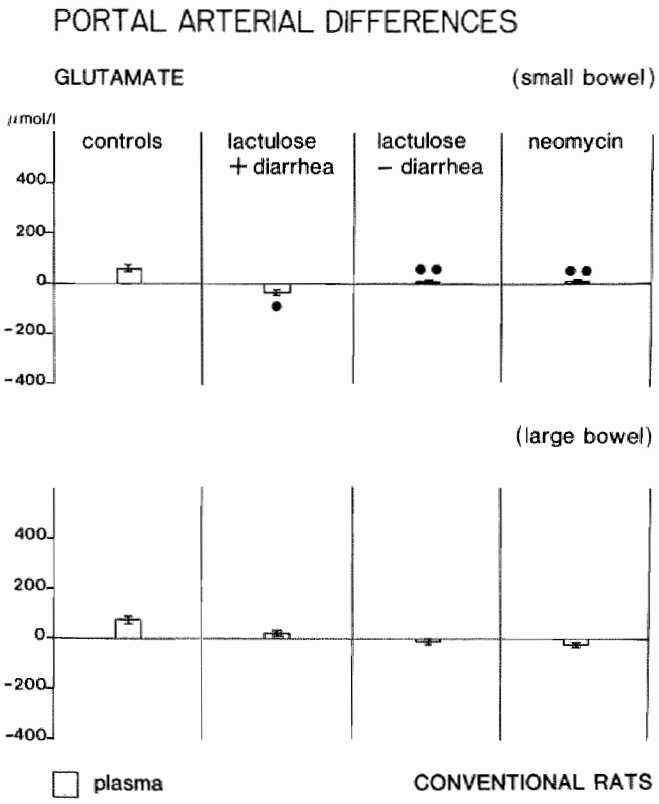


Figure 4: Portal-arterial glutamate concentrations in the small intestine and large intestine of conventional rats (fed state) measured in plasma of control, lactulose and neomycin treated rats. Positive and negative values represent output and uptake respectively in µmol/L (mean ± SEM). * $p < 0.05$; ** $p < 0.01$.

In the colon no decrease of glutamate production was observed after treatment with lactulose and neomycin. A net production of alanine (fig. 5) occurred in the small intestine which decreased significantly in the neomycin group and in plasma of the lactulose with and without diarrhoea group. The decrease in P.A. alanine concentration (table II) is mainly due to a decrease in the alanine portal vein concentration. Large intestine generates less alanine than the small intestine as expressed in the P.A. differences. Decreased plasma concentrations were noted after treatment with neomycin although no significance was reached. No decrease in alanine concentration was noted in the plasma of the lactulose with diarrhoea group in contrast to the lactulose without diarrhoea group, who showed an uptake.

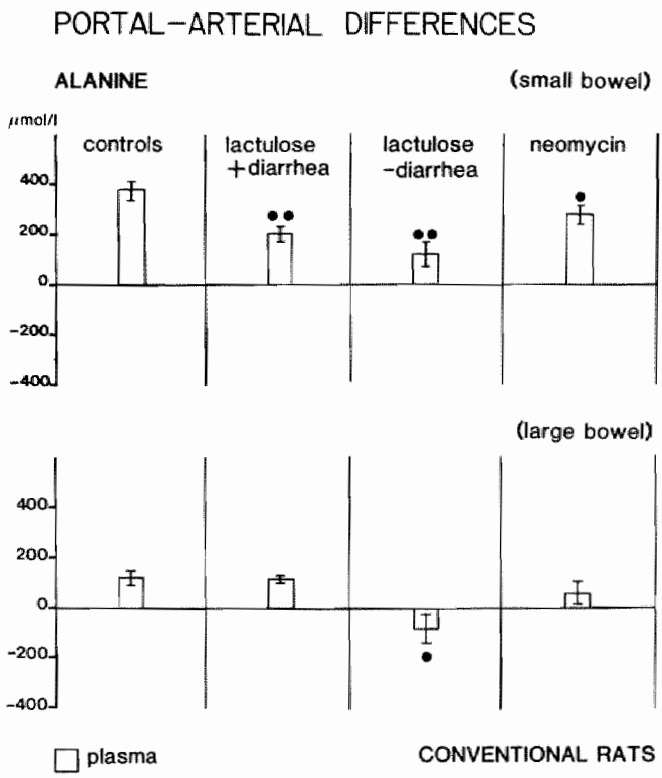


Figure 5: Portal-arterial alanine concentrations in the small and large intestine of conventional rats (fed state) measured in plasma of control, lactulose and neomycin treated rats. Positive and negative values represent output and uptake respectively in µmol/L (mean ± SEM).

* $p < 0.05$; ** $p < 0.01$.

In the small intestine (fig. 6) both lactulose and neomycin significantly decreased glutamine uptake. In the colon glutamine uptake was decreased in the lactulose with diarrhoea, lactulose without diarrhoea and neomycin group. No decrease was noted in the P-A differences of citrulline and ornithine.

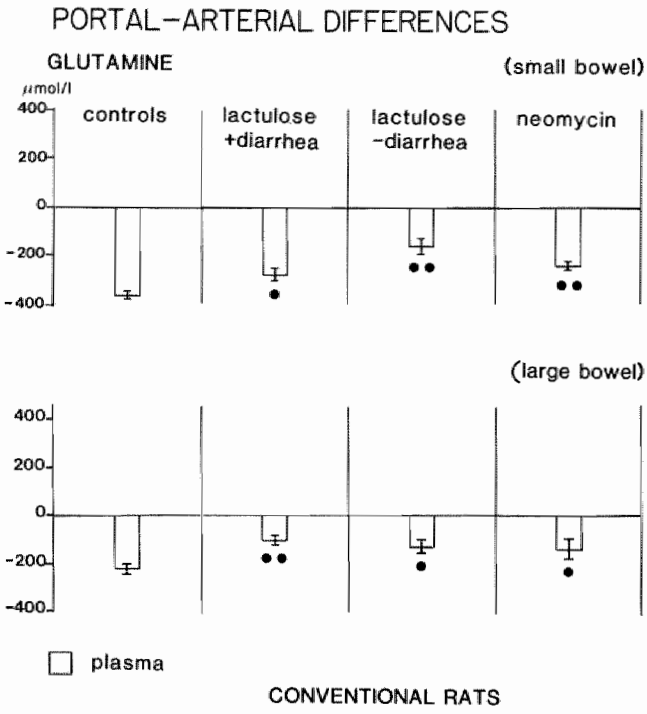


Figure 6: Portal-arterial glutamine concentrations in the small and large intestine of conventional rats (fed state) measured in plasma of control, lactulose and neomycin treated rats. Positive and negative values represent output and uptake respectively in µmol/L (mean ± SEM).

* $p < 0.05$; ** $p < 0.01$.

The influence of lactulose and neomycin on portal-arterial differences in germ-free rats.

In the germ-free animals after treatment with lactulose and neomycin we did not distinguish between portal blood of colon and small intestine but for economical reasons the combined portal effluent of small and large bowel was used. Stomach and spleen were excluded however. In the lactulose group only the animals with diarrhoea were studied. Lactulose significantly decreased the ammonia concentration in plasma in contrast to neomycin (fig. 7).

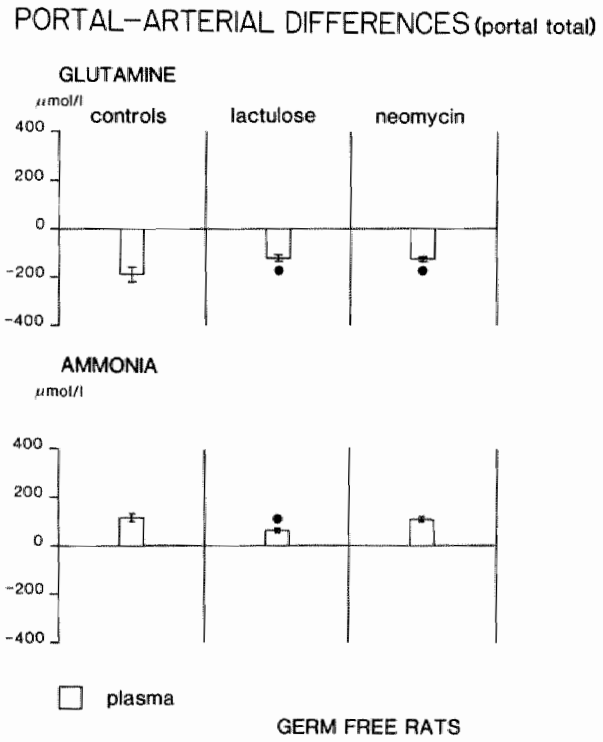


Figure 7: Portal-arterial ammonia and glutamine concentrations in the combined portal effluent of small and large intestine measured in plasma of control, lactulose and neomycin treated germ-free rats. Positive and negative values represent output and uptake respectively in μmol/L (mean ± SEM).

* $p < 0.05$; ** $p < 0.01$.

The glutamate P.A. difference was decreased after treatment with lactulose and neomycin in plasma (Fig. 8). Alanine production decreased in plasma after lactulose treatment (Fig. 8). Uptake of glutamine was decreased after treatment with lactulose and neomycin (Fig. 7). No decrease was noted in the P.A.-differences of citrulline and ornithine.

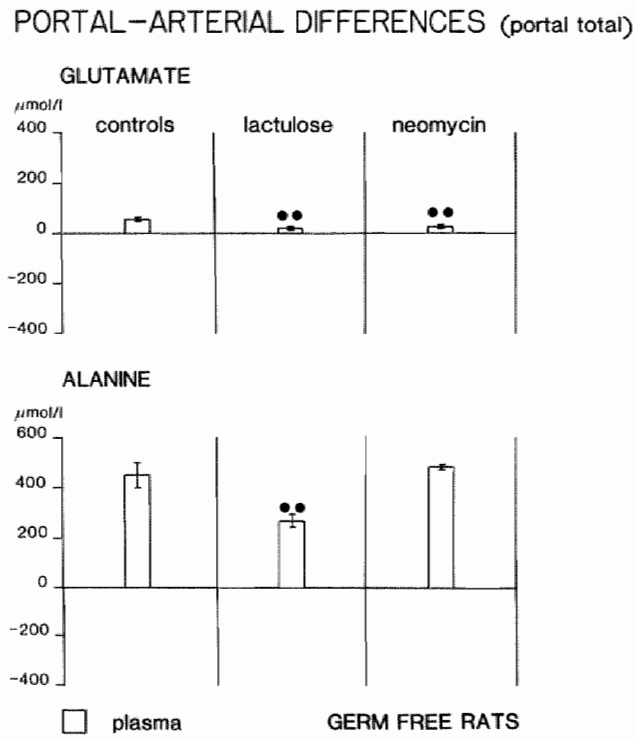


Figure 8: Portal-arterial alanine and glutamate concentrations in the combined portal effluent of small and large intestine measured in plasma of control, lactulose and neomycin treated germ-free rats. Positive and negative values represent output and uptake respectively in µmol/L (± SEM).

* $p < 0.05$; ** $p < 0.01$.

The P-A differences as demonstrated in fig. 1-8 were calculated from absolute values, as shown in tables 1-5.

Table I

	Controls Plasma	Lactulose + diarrhoea Plasma	Lactulose - diarrhoea Plasma	Neomycin Plasma
Ammonia	114.0 ± 13.6	106.6 ± 7.8	121.6 ± 16.0	81.2 ± 4.2
Glutamic acid	48.0 ± 3.0	101.2 ± 7.51	118.0 ± 14.5	118.8 ± 7.9
Alanine	326.0 ± 8.0	349.6 ± 23.1	485.0 ± 49.8	353.1 ± 21.8
Citrulline	68.0 ± 2.0	35.8 ± 2.74	60.5 ± 0.8	44.8 ± 6.06
Ornithine	52.0 ± 3.0	39.8 ± 2.13	55.0 ± 3.97	37.3 ± 5.15
Glutamine	697.0 ± 25.0	552.0 ± 22.6	548.7 ± 32.06	514.1 ± 14.98
	n = 15	n = 9	n = 9	n = 8

Table I: Arterial ammonia and amino acid concentrations in plasma of conventional rats (fed state). Arterial blood was collected from the aorta of controls, lactulose and neomycin treated rats. Results are expressed in µmol/L (± SEM).

Table II

	Controls Plasma	Lactulose + diarrhoea Plasma	Lactulose - diarrhoea Plasma	Neomycin Plasma
Ammonia	426.0 ± 35.8	270.0 ± 12.8	334.0 ± 45.7	135.3 ± 16.5
Glutamic acid	105.6 ± 8.1	67.6 ± 12.5	120.0 ± 14.5	110.6 ± 10.3
Alanine	709.4 ± 42.8	551.4 ± 21.2	605.3 ± 60.1	640.3 ± 63.2
Citrulline	110.4 ± 5.9	84.8 ± 7.2	81.8 ± 7.8	106.5 ± 11.3
Ornithine	64.4 ± 3.7	48.6 ± 2.8	59.5 ± 1.7	54.0 ± 2.5
Glutamine	330.6 ± 38.4	280.1 ± 23.9	393.6 ± 35.6	276.3 ± 12.7
	n = 5	n = 6	n = 6	n = 5

Table II: Portal ammonia and amino acid concentrations in the plasma of conventional rats (fed state). Portal concentrations were selectively measured in the portal effluent of the small intestine of controls, lactulose and neomycin treated rats. Results are expressed in µmol/L (± SEM).

Table III

	Controls Plasma	Lactulose + diarrhoea Plasma	Lactulose - diarrhoea Plasma	Neomycin Plasma
Ammonia	548.0 ± 57.4	408.0 ± 70.7	758.0 ± 56.7	237.2 ± 58.5
Glutamic acid	118.0 ± 11.0	121.6 ± 8.8	119.5 ± 11.7	108.4 ± 8.6
Alanine	452.0 ± 20.0	437.0 ± 25.9	357.0 ± 43.9	433.6 ± 23.5
Citrulline	86.0 ± 3.0	48.0 ± 3.5	46.5 ± 3.5	73.8 ± 1.3
Ornithine	65.0 ± 3.0	43.6 ± 3.9	56.0 ± 2.5	49.6 ± 1.2
Glutamine	447.0 ± 18.0	467.0 ± 11.2	403.0 ± 45.4	372.0 ± 42.2
	n = 5	n = 5	n = 5	n = 5

Table III: Portal ammonia and amino acid concentrations in the plasma of conventional rats (fed state). Portal concentrations were selectively measured in the portal effluent of the large intestine of controls, lactulose and neomycin treated rats. Results are expressed in µmol/L (± SEM).

Table IV

	Controls Plasma	Lactulose + diarrhoea Plasma	Neomycin Plasma
Ammonia	200.0 ± 12.45	136.0 ± 7.25	184.2 ± 10.98
Glutamic acid	126.2 ± 8.7	107.3 ± 6.67	118.7 ± 3.8
Alanine	873.2 ± 44.1	675.1 ± 21.0	904.2 ± 37.4
Citrulline	133.6 ± 8.0	118.8 ± 4.0	140.0 ± 11.67
Ornithine	62.2 ± 5.9	48.3 ± 2.9	70.2 ± 7.9
Glutamine	216.8 ± 33.7	140.3 ± 24	136.0 ± 3.0
	n = 5	n = 6	n = 5

Table IV: Portal ammonia and amino acid concentrations in plasma of germ-free rats (fed state). Portal concentrations were determined in the combined portal effluent of small and large intestine. Results are expressed in µmol/L (± SEM).

Table V

	Controls Plasma	Lactulose + diarrhoea Plasma	Neomycin Plasma
Ammonia	85.0 ± 3.7	68.6 ± 5.9	73.6 ± 4.2
Glutamic acid	89.1 ± 13.7	93.2 ± 3.9	72.3 ± 11.9
Alanine	412.1 ± 36.8	400.4 ± 19.1	377.2 ± 20.3
Citrulline	83.8 ± 0.9	73.0 ± 8.4	60.0 ± 8.0
Ornithine	50.8 ± 2.0	44.6 ± 2.4	52.8 ± 1.5
Glutamine	377.0 ± 8.7	281.0 ± 33.0	285.0 ± 19.5
	n = 6	n = 5	n = 5

Table V: Arterial ammonia and amino acid concentrations in plasma of germ-free rats (fed state). Arterial blood was collected from the aorta of controls, lactulose and neomycin treated rats. Results are expressed in µmol/L (± SEM).

VII.4. Discussion

In contradistinction with to the clinical literature, which still defines the colonic flora as the main producer of ammonia, this study clearly indicates that ammonia production in the small intestine is quantitatively more important than the large intestine. The small intestine produces more ammonia than the large intestine especially when a flow rate of small : large of 3 : 1 is taken into account (13).

Together with this ammonia production, alanine, citrulline, ornithine, proline and glutamic acid is formed. This production is not only bacterially mediated since germ-free rats also were capable of generating a significant amount of ammonia. Neomycin is supposed to act on the colonic flora thereby reducing ammonia levels (11). In this study however neomycin significantly decreased ammonia production in both the small and large intestine. That this ammonia lowering effect was due to a decrease in the aerobic flora is difficult to believe since the aerobic flora represents a small fraction of the total flora in the intestine and since the aerobic flora has been demonstrated not to contribute significantly to ammonia production (chapter V). Also relatively few bacteria are present in the small intestine when compared to the colon, where a lesser decrease was noted. Lactulose, like neomycin, is also supposed to act by interfering with the colonic flora. In this study however lactulose clearly interferes with intermediary metabolism in germ-free rats and therefore also in conventional rats. The precise mechanism of action of both neomycin and lactulose on glutamine metabolism is speculative. First of all one should keep in mind that this experimental model has its limitations. In this model, it was not possible to measure flow rates in the venous effluent of small and large intestine. Also the surgical procedure, which included ligation of arteries and veins and subsequently blood sampling from the vena porta, could have an impact on the determination. Keeping the limitations of this model in mind, there still is a consistent picture. Both lactulose and neomycin appear to be capable of interfering with intermediary

metabolism. Lactulose, if looking also at portal arterial differences of other amino acids like leucine, valine and tyrosine reduces uptake (data not shown). Most if not all amino acids shift into the direction of the lumen. This may be explained by an osmotic effect of lactulose. In this way glutamine availability for glutaminase activity is diminished, so that less ammonia is produced. Similarly neomycin causes amino acids to shift into the direction of the lumen, in this manner also reducing glutamine availability for glutaminase activity. More precise biochemical studies however are needed to reveal the precise mode of action of both drugs. Studies with a large animal model are needed to study ammonia and amino acid metabolism in different parts of the intestinal tract over longer periods of time and to study the influence of administration of lactulose and neomycin.

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Chapter VIII

GENERAL DISCUSSION

The results of this study indicate that large and small intestine generate ammonia. *In vitro* the amounts of ammonia produced by conventional and germ-free rats are comparable and are greatly increased when glutamine is supplied as a substrate. This signifies that *in vitro* gut production of ammonia in conventional rats is largely glutamine dependent and not mediated by bacterial action. From our studies it is clear that glutamine uptake and ammonia production occurs in both villous and crypt cells. Ammonia production in the small bowel *in vitro* is greater than in the colon. The difference in metabolic ammonia production *in vivo* should even be greater because the small bowel receives three times as much arterial flow and thus glutamine as the colon. Furthermore resorption of amino acids and thus substrate, occurs predominantly in the small bowel and to a much lesser extent in the colon. *In vivo* ammonia production in the small intestine was also quantitatively more important than in the large intestine. This production is to a large extent not bacterially mediated since also germ-free rats were capable of generating a significant amount of ammonia, and because the small intestine contains less bacteria if compared to the colon. In contradistinction with the *in vitro* situation however, *in vivo* germ-free rats produce less ammonia than conventional rats.

Why do conventional rats produce more ammonia than germ-free rats ?

As far as we can see there are two possible reasons:

1. In conventional animals bacteria contribute significantly to ammonia production.

2. In conventional animals arterial blood contains significantly higher concentrations of glutamine than in germ-free animals.

A higher concentration of substrate (glutamine) is therefore presented to the gut, which may result in increased uptake of glutamine and increased production of ammonia. Glutamine concentrations and similarly the glutamine/alanine ratio present in venous blood from the extremities have been shown to vary with different clinical states. In patients with liver cirrhosis for instance Imler has shown that the glutamine/alanine ratio greatly increases, compared to healthy controls. The same phenomenon was demonstrated when experimental animals were infused with ammonium acetate. Imler (1) attributes this to ammonia which is presented to the peripheral tissues in cirrhotic patients. This ammonia is "detoxified" by the formation of glutamine from glutamic acid. Glutamic acid is formed from alpha-ketoglutarate via a transamination reaction with branched chain amino acids. This mechanism has been claimed by Holm (2) to underlie the decrease in plasma branched chain amino acid levels encountered in patients with chronic liver disease or in experimental animals infused with ammonium salts.

In the studies described in chapter VII we found in germ-free rats an arterial glutamine/alanine ratio which was much lower than in conventional rats. Not only the ratio was lower however, but also the absolute arterial plasma levels of glutamine. In accordance with the mechanism postulated in patients with chronic liver disease or after ammonia infusions, the decreased glutamine/alanine ratio in germ-free rats may then be explained by decreased arterial NH_3 levels present in germ-free rats. It is very likely that this mechanism indeed is operative in germ-free rats and influences the glutamine/alanine ratio. The extent however of the increase of the glutamine/alanine ratio and of absolute glutamine levels in arterial blood in conventional rats compared to germ-free rats, appears to be much greater than can be explained by the only moderate increases in arterial ammonia levels in conventional rats (conventional rats: 85 μmol/L; germ-free rats: 115 μmol/L). The large extent of the increase in arterial glutamine levels in conventional rats

needs therefore further investigation. There are several possibilities:

1. The extraction rate of ammonia in muscle is possibly increased, resulting in higher glutamine production than would seem appropriate when only the slight excess of arterial ammonia in conventional rats would be taken up and transformed into glutamine. Venous NH_3 levels were not measured however.
2. Another possibility is that glutamine production is enhanced by ammonia out of proportion with the slight increase in arterial ammonia. Ammonia might for instance influence transport or enzyme systems, in turn influencing the glutamine/alanine ratio. Indeed our data show that the increase of glutamine occurs at the expense of alanine in conventional rats and vice versa in germ-free rats (chapter VII, tabel V).
3. Also other influences can not be ruled out. Kipnis and his group (3) demonstrated that hormones (epinephrine, thyroxine) and amino acids can influence the glutamine/alanine ratio as released in vitro by rat epitrochlearis muscle. It is unlikely that germ-free rats have hormonal and amino acid patterns that differ substantially from conventional rats. It would however seem to be more relevant to study substances like Interleukin I released by macrophages operative in the gut in the defense against bacteria.

In conclusion our data do not allow precise quantitation of bacterial and metabolic ammonia generation. Weber (4) has concluded from his experiments in dogs that almost all ammonia production in the small bowel can be accounted for by glutamine dependent metabolism in the mucosa. In the colon half of the ammonia production was claimed to be glutamine dependent. Taking these data together, considerably less than 25% of total ammonia production in the gut would be bacterially mediated because the colon receives less flow than the small bowel. Still total ammonia production in the gut of conventional rats is roughly a factor 2-3 greater than in germ-free rats. We suggest that the presence of bacteria in the gut increases ammonia in the portal vein which is

largely taken up by the liver. A small part of it escapes however into the general circulation where it influences the glutamine/alanine ratio disproportionately, i.e. above the amount required to clear the excess of "toxic" arterial ammonia. The mechanisms underlying this process are not entirely clear. The increase in systemic glutamine levels has been shown (chapter VII) to result in increased uptake and degradation in the gut mucosa. In the degradation process of glutamine at least 80% of the amide nitrogen of glutamine is released into the portal vein as ammonia.
Therefore the presence of bacteria in the gut influences whole body nitrogen in such a way that much more ammonia is produced in the gut than can be accounted for by bacterial action alone.

How relevant are these findings in terms of whole body nitrogen metabolism ?

Ninety percent of all nitrogen resulting from muscle proteolysis is released into the systemic circulation, not as the constituting amino acids, but as glutamine and alanine. This occurs in most organs, notably skeletal muscle, heart muscle, adipose tissue and brain. To our knowledge it has not been the subject of study what happens with muscle tissue in the gut and with protein in the kidney, lungs, etc. In addition all these tissues (skeletal muscle, cardiac muscle, brain) can take up ammonia, which results in glutamine formation. Therefore products of protein degradation in the body are largely released into the circulation as glutamine and alanine. As discussed before the ratio between glutamine and alanine varies from 3 : 2 in normal humans to 2 : 1 (chapter VII) in conventional rats in this study. To achieve homeostasis the glutamine and alanine produced have to and can only be taken up in the gut and in the kidney. It has been demonstrated that the liver can take up glutamine but does so only to a very limited degree. Alanine is of all amino acids the major precursor for gluconeogenesis and urea genesis in the liver. Glutamine therefore has to be taken up largely by the gutmucosa and the kidney. The first step in the degradation of glutamine is deamidation

which results in ammonia production. Some 80 percent of this ammonia is released into the portal circulation. Only a minor part is directed into citrulline synthesis. In the kidney glutamine degradation is quantitatively less important but ammonia is also produced and then excreted into urine as long as urinary pH is low.

The use of such a set up is evident! The bulk of the products of peripheral protein degradation gets access to the systemic circulation as glutamine and alanine. Glutamine then acts as a non-toxic carrier of two nitrogen atoms per molecule of glutamine, and is almost exclusively taken up by gutmucosa and kidney. Ammonia is produced in the gut and released into the portal circulation where it can almost completely be cleared by the liver in a first pass, so that very little escapes into the systemic circulation. Similarly ammonia derived from glutamine degradation in the kidney can be excreted in a harmless way in an acid urine, so that it does not get access into the general circulation (and thus the brain). In addition it may also be useful that the major part of amino acids do not escape into the general circulation as such because they also may have harmful and even toxic effects, if present in excess.

From the chain of events described above, one can roughly estimate that 20-30% of the nitrogen excreted per day in the urine is glutamine derived ammonia, produced in the gut and presented to the liver where it is transformed into urea. This means that, if we assume that in a normal grown-up person 10 g of urinary nitrogen is excreted per day, 2-3 g is derived from metabolic ammonia produced in the gut, equalling to 143-214 mMol of ammonia.

It has been hypothesized that catabolism precipitates hepatic encephalopathy by the release of amino acids derived from the breakdown of endogenous protein (5). The hypothesis was based upon the observation that disease, shock and other catabolic insults, in clinical practice frequently induce hepatic encephalopathy, and upon the amino acid neurotransmitter hypothesis which claims that a distorted amino acid pattern influences brain neurotransmitter metabolism, resulting in hepatic encephalopathy.

The amino acid neurotransmitter hypothesis was modified however and linked to ammonia metabolism (chapter I). The biochemical literature and the results of this thesis provide further insight in potential mechanisms underlying the encephalopathy precipitating effect of catabolic states. Similar as in the healthy organism increased breakdown of peripheral protein presents the organism with glutamine and alanine, albeit in increased amounts. Likewise increased glutamine flow results in increased ammonia production in the gut, which either due to diminished hepatic metabolic function or due to collateral circulation, gets access to the systemic circulation and consequently to the brain.

Another clinical observation is that different types of dietary protein have different influences on hepatic encephalopathy. Blood appears to be more toxic than meat and meat more toxic than vegetable protein. Blood contains much more glutamine than whole protein, but we are not aware of studies establishing the glutamine content of meat, milk, fish, vegetables etc. It may be worthwile to explore this matter in more detail.

Effects of lactulose and neomycin.

Our first studies (chapter II, chapter III) demonstrate the likelihood that lactulose directly interferes with the uptake of glutamine and its subsequent metabolism. Studies regarding the potential influence of lactulose on bacterial flora did not reveal major changes in bacterial counts. Lactulose may however have interfered with uptake of nitrogen containing products of bacterial metabolism, including ammonia, from the lumen across the gutwall into the portal blood. In these studies we did not measure total nitrogen and ammonia in the stools of these rats however. Study of arterial and portal concentrations of amino acids and ammonia in germ-free and conventional rats *in vivo* reveals that lactulose interferes with glutamine uptake which results in a diminished release of ammonia, alanine, glutamic acid etc. into the portal blood, especially in the small bowel. These findings prove that

lactulose via this action significantly inhibits ammonia generation in the gut mucosa. As already mentioned, the discrepancy between estimated ammonia production by bacteria (less than 25%) (4) and the 2-3 fold increase in total ammonia production in conventional rats compared to germ-free rats has been explained by the change in venous c.q. arterial glutamine/alanine ratio in some way brought about by the presence of bacteria in the gut. Lactulose appears to interfere slightly with the ratio by decreasing arterial glutamine levels, but alanine levels remain constant. It is therefore unlikely that lactulose influences the glutamine/alanine ratio of degradation products of peripheral proteins like muscle. Rather it appears that it exerts its action at the bowel wall itself for two reasons:

1. Diarrhoea should be present to achieve the full lactulose effect suggesting that its actions takes place predominantly in the gut.
2. Study of the full pattern of amino acids across the bowel wall reveals that lactulose inhibits resorption from the gut lumen of all amino acids including glutamine or/and even induces a shift of glutamine and other amino acids from the gutwall to the gut lumen (data not shown). The moderate decline in the glutamine/alanine ratio therefore appears to be the result of diminished uptake from or release into the gut lumen rather than of interference with the composition of degradation products of peripheral protein.

The action of neomycin is even more of an enigma. Its antibacterial effect is exclusively directed against the enterobacteriaceae, which constitute only a minute part of the total flora, and which have been shown in our studies to produce negligable amounts of ammonia. Still we can not exclude the possibility, and even think that it is probable, that neomycin interferes with the way products of bacterial metabolism are handled in the gut, and that neomycin changes the fate of these products, possibly in a similar way as the metabolism of the gut mucosa is influenced. Following the same line of reasoning as with lactulose our data suggest that neomycin interferes with

uptake of glutamine by the gutwall, either by diminishing uptake from the lumen, or/and by diminishing uptake from the arterial blood. Portal arterial differences even suggest that neomycin induces a shift of all amino acids from the gutwall to the gut lumen, albeit smaller than is achieved by lactulose. Extra evidence is derived from morphological data described in chapter IV. Although qualitative rather than quantitative in nature this investigation provides evidence that neomycin interferes with mucosal enzyme systems and therefore most likely also interferes with aspects of mucosal metabolism. It should be realized that the predominant importance that is attached by clinicians to bacterial metabolism in the pathogenesis of hyperammonaemia and hepatic encephalopathy has resulted from two factors with paramount influence.

1. Dame Sheila Sherlock herself emphasized the role of bacteria (6).
2. The importance of bacteria was derived from the observation that antibiotics (chlortetracyclin) ameliorated hepatic encephalopathy.

Of the more general anti"biotic" actions of antibiotics too much attention was concentrated on their anti"bacterial" action resulting in the conviction of clinicians that antibiotics exert an exclusive action on bacteria without at all interfering with metabolism of the tissues of the host. In other areas of medicine however nowadays it is appreciated that bacteria can have important toxic and even deleterious effects on host tissues including the gut. Furthermore lack of contact between clinical and basic science allows misunderstandings to persist in one area of the scientific world despite more advanced knowledge in other areas. This problem has been extensively illustrated for glutamine metabolism and ammonia generation in the introductory chapter of this thesis. At present the same phenomena can be observed for -amino-butyric acid (GABA), believed to have an important role in the genesis of acute hepatic encephalopathy due to acute hepatic failure. GABA again is believed by clinicians to be generated in the gut by bacterial action, whereas the biochemists know that the gutmucosa contains all enzymes to produce GABA. It would not surprise us at all if further study would show

that GABA is produced in the gutwall, increases after meals and is glutamine dependent.

Areas for future research.

We are aware of the crude nature of a part of our experiments, specifically the sampling of portal effluent of small and large bowel in a semi in vivo situation (living animal, open abdomen, clamping of certain areas of the gut, and sampling of amounts of blood that induce hypovolemia during the taking of the second sample).

All the results point into one direction however which warrants further investigations in a larger animal, preferably in a non stressed condition. Furthermore this thesis does only provide circumstantial evidence with regard to the mechanisms involved in the actions of neomycin and lactulose on the gutwall. In addition factors (ammonia and others) influencing the arterial glutamine/alanine ratio should be better defined.

Finally because of recent interest in the GABA hypothesis and because of the possibility that glutamine degradation can substantially contribute to GABA generation in the gut, this area needs further study.

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SUMMARY

In chapter I the literature is reviewed concerning the mechanism involved in ammonia generation in the gut. Apart from bacterial ammonia generation, there are indications that there is a substantial ammonia production in the mucosa of the jejunum, ileum and colon, in that order of magnitude. This metabolic ammonia production is glutamine dependent. Ammonia still figures in most theories concerning the pathogenesis of hepatic encephalopathy (H.E.). The relevance of this study is underlined by a review of the literature demonstrating that neomycin and lactulose are indeed effective in ameliorating H.E., probably by their ammonia decreasing effect, although a well designed double-blind trial with a placebo has never been performed. The experiments described in this study were undertaken to establish if lactulose and neomycin interfere with mucosal ammonia metabolism, since this effect has never been considered in their mode of action. Current explanations about the mode of action of lactulose and neomycin are contradictory and unsatisfactory.

As a first approach to the problem we performed incubation studies with intact pieces of small and large intestine in a Krebs-Ringer solution (with and without glutamine) and with lactulose and neomycin added to the incubation medium (chapter II). Small and large intestine were capable of metabolizing large quantities of glutamine. Small intestine produced per unit of weight more ammonia than large intestine when glutamine was added. Lactulose inhibited degradation of glutamine in a dose dependent manner. Theoretically the muscle in the gut could have a major effect on metabolism of ammonia. Therefore we carried out experiments, as described in chapter III, with isolated mucosa cells to pinpoint the site where ammonia metabolism occurs. In addition these studies allowed us to distinguish between ammonia production of villous and crypt cells. Furthermore the effect of neomycin and lactulose was investigated. Isolated cells were prepared according to the

method of Harrison and Webster. Results indicated that glutamine is metabolized by both villous and crypt cells and that ammonia, glutamic acid and alanine is produced. Both lactulose and neomycin were capable of inhibiting the alanine dependent production of ammonia by villous and crypt cells of conventional and germ-free rats. This glutamine degradation appears not to be mediated by bacterial action since processing these mucosa cells does not allow bacteria to stay on the surface of the mucosa cells. Furthermore mucosa cells of germ-free rats were capable of generating comparable amounts of ammonia as cells of conventional rats in both villous and crypt cells.

In a separate study (chapter IV) we evaluated the effect of neomycin on gut-morphology with several histochemical staining reactions. Neomycin diminished both in normal and germ-free rats the activity of NAD-tetrazolium-reductase (NADHtr), succinate dehydrogenase (SDH), alkaline phosphatase (AlPh) and acid phosphatase (AcPh). The findings of this study indicate that explanations for the benefical effects of neomycin on hyperammonemia in liver disease should not only include the antimicrobial activity of neomycin but also its influence on absorptive and metabolic function of the mucosa cell.

With the study described in chapter V we tried to establish, which bacteria are responsible for ammonia production, and if bacteria are responsible. Therefore rats were selectively decontaminated, which means that a defined portion (aerobic or anaerobic) was selectively eliminated by the use of specific antibiotics. Elimination of the anaerobic flora decreases, in contrast to statements in the literature, portal ammonia levels. The study however does not prove that elimination of the anaerobic flora is largely responsible for the decrease in portal ammonia concentration because the antibiotics used may apart from the bactericidal action, also exert metabolic effects. We therefore created gnotobiotic rats with a defined flora. In these rats ammonia was determined in the portal blood and related to the flora present in the intestine. After colonization of germ-free rats with anaerobic or aerobic intestinal flora or a combination of both, portal plasma levels increased in the anaerobic, combined anaerobic and aerobic, but not in the

aerobic gnotobiotic animals. Although the anaerobic flora consisting of Clostridum perfringens, Clostridium inocuum, Bacteroides ruminiculum, Bacteroides fragilis, Pepto-streptococci, Bifido bacteria and Lactobacilli, generates a significant amount of ammonia, the portal ammonia concentration did not reach levels of conventional rats, suggesting that additional, possibly unknown unclassified species may be needed to restore ammonia levels in the portal vein to plasma levels in the portal vein of conventional rats. In chapter VI studies are described concerning the influence of lactulose and neomycin on the luminal and gutwall associated flora of both the small and large intestine. The changes in flora were correlated with the concentration of ammonia in the portal effluent of small and large intestine. The bacterial counts in small and large intestine of the rats after treatment with lactulose and neomycin cannot explain the significant decrease in ammonia levels in the portal vein. Consequently, the mechanism of action of lactulose and neomycin can not be exclusively explained by these changes in the gut flora, especially not since both neomycin and lactulose also decrease ammonia levels in the portal effluent of the small intestine where few bacteria are present. Finally (chapter VII) we determined portal and arterial amino acid and ammonia concentrations and calculated portal-arterial differences in small and large bowel of control animals and of lactulose and neomycin treated rats. Both conventional and germ-free rats were used. Under these in vivo conditions colon and small bowel take up glutamine and produce alanine, glutamate and ammonia. Small intestine releases more alanine and ammonia than the colon especially when a flow rate of small versus large bowel of 3 : 1 is taken into account. Glutamine uptake and production of alanine, glutamate and ammonia were decreased by lactulose and neomycin in small and large bowel. In chapter VIII these results are discussed.

SAMENVATTING

In hoofdstuk I wordt een literatuuroverzicht gegeven betreffende de mechanismen die een rol spelen bij de ammoniak productie in de darm. Buiten het feit dat er ammoniak gegenereerd wordt door de bacteriën, zijn er aanwijzingen dat er een belangrijke hoeveelheid ammoniak gemaakt wordt in de mucosa van het jejunum, ileum en colon in die volgorde van grootte. Ammoniak wordt nog steeds genoemd in de hypotheses van het hepatisch coma. De relevantie van deze studie wordt onderbouwd door een literatuuroverzicht dat aangeeft dat zowel lactulose als neomycine hun effect hebben in verbetering van het coma, hoogstwaarschijnlijk door hun ammoniakverlagende effect. Een goed dubbelblind onderzoek met een placebo is echter tot op heden nooit uitgevoerd. De studies beschreven in dit proefschrift werden gedaan om na te gaan of lactulose en neomycine hun invloed uitoefenen op het intermediair metabolisme in de darm, gezien het feit dat deze mogelijkheid nog nooit onderzocht is. De algemeen gangbare verklaringen omtrent de werking van lactulose en neomycine zijn contraversiëel en onbevredigend.

Als een eerste aanzet tot het probleem werden incubatie studies gedaan met intacte stukjes weefsel van dunne en dikke darm in een Krebs-Ringer oplossing (met en zonder glutamine) en met lactulose of neomycine toegevoegd aan het incubatiemedium (hoofdstuk II). Dunne en dikke darm bleken in staat te zijn om grote hoeveelheden glutamine te metaboliseren waarbij de dunne darm meer ammoniak produceerde wanneer glutamine werd toegevoegd aan het medium. Lactulose remde de degradatie van glutamine op een concentratie afhankelijke manier. Gezien het theoretische feit dat de spier een invloed zou kunnen uitoefenen op deze ammoniakproductie werden experimenten gedaan zoals beschreven in hoofdstuk III met geïsoleerde mucosacellen, om de plaats van de ammoniakproductie vast te stellen. Tevens gaf dit ons de mogelijkheid de ammoniakproductie van zowel de villus als de crypt te bestuderen en te vergelijken. Bovendien werd het effect van lactulose en neomycine op deze ammoniakproductie bestudeerd.

Mucosacellen werden geïsoleerd volgens de methode van Harrison & Webster. De resultaten gaven aan dat zowel villus als crypt cellen beiden in staat waren glutamine te metaboliseren tot ammoniak, glutaminezuur en alanine. Zowel lactulose als neomycine remden de glutamine afhankelijke ammoniakproductie in de villus en cryptcellen van steriele en conventionele ratten. De afbraak van deze glutamine blijkt volledig onafhankelijk te zijn van bacteriën, daar ten eerste bij deze methode van isoleren vrijwel geen bacteriën achterblijven en ten tweede mucosacellen van steriele ratten ook in staat bleken te zijn vergelijkbare hoeveelheden ammoniak te maken zowel in de villus als in de crypt.

In hoofdstuk IV wordt het effect bestudeerd van neomycine op de morfologie van de darm door middel van verschillende enzymkleuringen. Neomycine bleek zowel bij steriele als bij conventionele ratten de activiteit van NAD tetrazoliumreductase, succinaat-dehydrogenase, alkalische en zure fosfatase te remmen. De resultaten van deze studie suggereren dat de verklaringen omtrent de werking van neomycine bij het hepatisch coma, niet alleen door de antimicrobiële werking verklaard kan worden, maar ook door een mogelijk effect op resorbtie, transport c.q. intermediair metabolisme.

Met de studie in hoofdstuk V hebben we geprobeerd na te gaan of bacteriën verantwoordelijk zijn voor de ammoniakproductie in de darm en zo ja, welke. Daartoe werden ratten selectief gedecontamineerd hetgeen inhoudt dat bepaalde groepen bacteriën (aerobe of anaerobe) geëlimineerd werden met antibiotica. Deze veranderingen werden gerelateerd aan de ammoniak concentratie in de vena porta. Eliminatie van de anaerobe flora verminderde, in tegenstelling tot hetgeen beweerd wordt in de literatuur, de ammoniak concentratie in de vena porta. Dit deel van de studie bewijst echter niet dat de anaerobe flora verantwoordelijk is voor de ammoniakproductie daar de gebruikte antibiotica tevens invloed zouden kunnen hebben op het niet bacterie gerelateerde metabolisme van de darm. Daartoe werden steriele ratten gecoloniseerd met een vooraf vastgestelde bekende flora (gnoto-bionten). Bij deze gnotobionten werd de ammoniakproductie in de vena porta gerelateerd aan de bacteriële inhoud

van de darm. Na colonisatie met een anaerobe of aerobe flora, of een combinatie van beiden, werd een stijging waargenomen van ammoniak in de vena porta in de anaerobe, gecombineerd anaerobe + aerobe, echter niet in de aerobe gnotobionten. Ondanks het feit dat de anaerobe flora de volgende species bevatte: Clostridium perfringens, Clostridium inocuum, bacteroides ruminiculum, bacteroides fragilis, pepto-streptococcen, bifido bacillen en lactobacillen, waardoor een aanzienlijke ammoniak stijging optrad, bereikte de uiteindelijke concentratie in de vena porta niet die van de conventionele ratten, hetgeen betekent dat andere, nog nader te specificeren of nooit eerder gekweekte soorten nodig zijn om de fysiologie te herstellen in de darm.

In hoofdstuk VI wordt een studie beschreven betreffende de invloed van lactulose en neomycine op de luminale en darmwand geassocieerde flora van zowel de dunne als de dikke darm. Deze floraveranderingen werden gecorreleerd aan de ammoniak concentratie in de vena porta. De resultaten geven aan dat de veranderingen van deze flora ten gevolge van de behandeling met neomycine en lactulose niet gerelateerd kunnen worden aan de ammoniakdalingen in de vena porta zodat de werking van neomycine en lactulose niet verklaard kan worden uit deze veranderingen temeer daar zowel lactulose als neomycine ammoniakdalingen te zien gaven in de dunne darm waar relatief weinig bacteriën aanwezig zijn.

Tenslotte bepaalden we in hoofdstuk VII de ammoniak- en aminozuurconcentraties in arteriëel en portaal bloed bij met lactulose en neomycine behandelde ratten. Zowel steriele als conventionele ratten werden gebruikt. Vanuit deze waarden werden portale-arteriële verschillen berekend. In deze in vivo studies blijkt zowel het colon als de dunne darm glutamine op te nemen en ammoniak, glutamine, alanine, citrulline en ornithine te produceren. De dunne darm produceert duidelijk meer ammoniak, zeker als men ook de grotere flow naar de dunne darm dan naar de dikke darm in acht neemt.

De opname van glutamine en de productie van alanine, glutaminezuur en ammonia werden zowel in de dunne als dikke darm geremd.

In hoofdstuk VIII worden deze resultaten besproken.

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CURRICULUM VITAE

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